Macrophage Inflammatory Protein-1α: An Inhibitor Of Clonogenic Epidermal Keratinocyte Proliferation?

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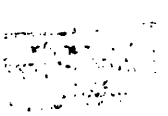


Contents.

Acknowledgements		ii
Table Of Contents		iii
Section 1	Introduction.	1
Section 2	Materials and Methods.	90
Section 3	Results.	117
Section 4	Discussion.	181
Section 5	References.	203

Section 1.
Introduction.

1.1. Haemopoiesis.	**1**
1.1.1.Stem Cells.	2
1.1.2. Progenitor Cells.	6
1.1.3. Mature Terminally Differentiated cells.	7
1.2. The Regulation of Haemopoiesis.	**8**
1.2.1. **The Stroma.**	**8**
1.2.2. **Growth Factors.**	**9**
1.2.2.1. Proliferation.	9
1.2.2.2. Differentiation.	10
1.2.2.3. Apoptosis.	10
1.2.2.4. The *in vivo* Evidence.	11
1.2.2.5. Functional Redundancy?	12
1.2.3.**Inhibitors.**	**12**
1.2.3.1. Tetrapeptide Inhibitor:AcSDKP.	14
1.2.3.2. Pentapeptide Inhibitor.	14
1.2.3.3. Tumour Necrosis Factor- α.	15
1.2.3.4. Interferons.	16
1.2.3.5. Transforming Growth Factor -β.	18
1.2.3.6. MIP-1α And The Chemokines.	23
1.3. The Chemokines.	**23**
1.3.1 The CXC Chemokines.	**24**
1.3.1.1. Platelet Factor -4.	24
1.3.1.2. Platelet Basic Protein.	25
1.3.1.3. γ-IP-10.	26
1.3.1.4. Interleukin -8.	27
1.3.1.5. GRO -α,β,γ.	29
1.3.1.6. The Stromal Derived Factors-1α and β	30
1.3.1.7. Other CXC Chemokines.	31
1.3.2. **The CC Chemokines.**	**32**
1.3.2.1. Macrophage Inflammatory Proteins	32
1.3.2.2. The Monocyte Chemotactic Proteins.	32
1.3.2.3. Eotaxin.	34
1.3.2.4. Rantes.	35
1.3.2.5. Myeloid Progenitor Inhibitory Factors.	36
1.3.2.6. Other CC chemokines.	36

1.3.3. **Macrophage Inflammatory Proteins.** **39**
1.3.3.1. The MIP-1α Gene And Its Expression. 39
1.3.3.2. The MIP-1 Proteins. 41
1.3.3.3. Aggregation. 42
1.3.3.4. Structure-Function Studies. 43
1.3.3.5. Receptors. 50
1.3.3.6. Signal Transduction. 52
1.3.3.7. MIP-1α And The Immune Response. 53
1.3.3.8. MIP-1α: The Regulation Of Cell Proliferation. 54
a) *Haemopoiesis.*
b) *Spermatogenesis*
c) *Clonogenic Keratinocytes.*

1.4. **The Epidermis.** **59**
1.4.1 **The Keratinocyte.** **60**
1.4.1.1. The Basal Layer. 60
1.4.1.2. The Stratum Spinosum. 69
1.4.1.3. The Stratum Granulosum. 70
1.4.1.4. The Stratum Corneum. 71
1.4.2. **Non-Keratinocyte Epidermal Cells.** **71**
1.4.2.1 The Melanocyte. 72
1.4.2.2 The Merkel Cell. 72
1.4.2.3 The γδ T–cell. 73
1.4.2.4 The Langerhans cell. 74
a) *Epidermal Development And TheLangerhans Cell.*
b) *Langerhans Cell Distribution.*
c) *The Langerhans Cell And The Immune Response.*
d) *The Langerhans Cell And The Regulation Of Keratinocyte Proliferation.*

1.5. **Regulators of Epidermal Keratinocyte Proliferation.** **79**
1.5.1. **Positive Regulators.** **79**
1.5.2. **The EGF Family.** **79**
1.5.3. **Non-EGF Family Mitogens.**
1.5.1.2 Keratinocyte Growth factor. 83
1.5.1.3 Other positive regulators. 85
1.5.2. **Negative Regulators.** **85**
1.5.2.1. TGF-β. 85
1.5.2.2. TNF-α. 87
1.5.2.3. Epidermal Pentapeptide. 87
1.5.2.4. MIP-1α. 88

AIMS OF THESIS. **89**

SECTION 2. **90**
MATERIALS AND METHODS.

Materials.
2.1 Tissue Culture Supplies. 90
2.2 Bacterial Hosts and Media. 91
2.3 Molecular Biology. 91
2.4 Protein Biochemistry. 92
2.5 Chemicals. 93
2.6 Solutions. 93

Methods.

2.7 Cell Culture. 95
2.8 Mice. 97
2.9 Harvesting of Bone Marrow. 97
2.10 The *in-vitro* CFU-A assay. 97
2.11 Recombinant DNA Techniques. 98
2.11.1 Host cells. 98
2.11.2 Transformation of bacterial host. 98
2.11.3 Bacterial glycerol stocks. 99
2.11.4 Preparation, Manipulation and Purification of Plasmid DNA 99
2.11.5 Engineering DNA Fragments by PCR. 102
2.11.6. Sequencing Of Plasmid DNA. 108
2.12.Introduction Of Recombinant Vectors into Mammalian Cells. 109
2.13. MIP-1α Protein Purification and Preparation for Bioassays. 110
2.14. Protein Characterisation. 111
2.14.1. SDS-PAGE gel electrophoresis. 111
2.14.2. Silver staining. 113
2.14.3. Western Blotting. 113
2.14.4. TGF-β1 quantitation using ELISA. 114
2.15.^{125}I Labelling of MIP-1α Protein. 115
2.16. Electrospray Mass Spectroscopy. 115

Section 3.
Results. **117**
3.1. Introduction. **117**
3.1.1. The Preparation Of Lab MIP-1α. 117
3.1.2. The Activity Of Lab MIP-1α On CFU-A Colony Formation. 119
3.1.3. Effects Of Lab MIP-1α On Keratinocyte Colony Formation. 119
3.1.4. The Inhibitory Activity Of Lab MIP-1α Is Reversed By A Specific Polyclonal Antibody To Bacterial Murine MIP-1α. 124
3.1.5. Summary. 124

3.2. Putative Accessory Factors. **126**
3.2.1 Proteoglycans. 126
3.2.2 Do COS 7 Cells Secrete A Co-Factor For The HEK Inhibitory Activity Of MIP-1α? 128
3.2.3 Summary. 135

3.3. Purification. **135**
3.3.1. Further Purification Of GI MIP-1α. 136
3.3.2. The Activity Of B2 Is Reversed By Specific Anti-MIP-1α Polyclonal Antibody. 140
3.3.3. Why Is The B2 MIP-1α Preparation Not As Potent As COS MIP-1α? 140
3.3.4. Does B2 Contain Low Levels Of Other Keratinocyte Inhibitors? 143
3.3.5. Summary. 144

3.4. The Activity Of Human MIP-1α Derived From A Stably Transfected CHO Cell Line. **146**

3.5. Does Some Form Of Structural Alteration Activate MIP-1α? **148**
3.5.1. Protein Sequencing. 148
3.5.2. Amino-Terminal Truncated Mutants. 151
3.5.3. Electrospray Mass Spectroscopy. 152
3.5.4. Activation By Proteolytic Cleavage. 157
3.5.5. Summary. 159

3.6.Reverse Phase Chromatography. **161**
3.6.1.Summary. 162

3.7.Amino-Terminally Truncated MIP-1α Mutants. **169**

SECTION 4. **177**
DISCUSSION.
4.1. The Activity Of Heparin Purified COS MIP-1α. 177

4.2. The Keratoinhibitory Activity Of COS MIP-1α: Co-Factor Or Structural Activation? 178

4.3. Purified COS MIP-1α Is Still Active. 179

4.4. The Structural Activation Of MIP-1α. 180

4.5. The Amino-Terminus In The Chemokine Family Of Proteins. 181

4.6. MIP-1α Signal Peptidase And Amino-Terminal Truncation. 182

4.7. Amino-Terminally Truncated MIP-1α And Keratinocyte Proliferation. 186

4.8. Systematic Amino-Terminal Truncation Of MIP-1α Molecule. 188

4.9. Potential Alternative Activating Mechanisms. 190

4.10. Future Studies. 192

4.11. Potential Clinical Relevance of MIP-1α. 197

Section 5.
REFERENCES **199**

FIGURES.

Figure 1a 118
A Silver Stained SDS-PAGE Gel of LAB MIP-1α.
Figure 1b 120
A Silver Stained SDS-PAGE Gel of GI and LAB MIP-1α.
Figure 1c 121
Both Bacterial and LAB MIP-1α inhibit the CFU-A assay.
Figure 2 122
LAB H2 and GI H2 both inhibit clonogenic keratinocyte proliferation.
Figure 3 123
Human epidermal keratinocyte colonies in KGM assay.
Figure 4 125
Anti-MIP-1α antibody reverses the activity of LAB MIP-1α.
Figure 5 127
Bacterial MIP-1α is not activated by protoglycans.
Figure 6 129
COS 7 cells do not constitutively secrete an accessory factor for MIP-1α.
Figure 7 131
The incubation of MIP-1α with COS 7 cells does not generate kerato-inhibitory activity.
Figure 8 133
COS 7 cells do not secrete an accessory factor for MIP-1α during transient transfection.
Figure 9 134
The addition of bacterial MIP-1α to low concentrations of GI MIP-1α does not significantly alter the activity of the active preparation.
Figure 10 137
Silver-stain of purified COS 7 MIP-1α.
Figure 11 138
Autoradiograph of B2 MIP-1α.
Figure 12 139
B2 is still able to inhibit keratinocyte proliferation.
Figure 13 141
Specific polyclonal antibody to MIP-1α is able to reverse the activity of B2.
Figure 14 142
4.5M $MgCl_2$ reduces the activity of GI MIP-1α.
Figure 15 145
The effect of the TGF-β latency associated peptide LAP on the activity of GI MIP-1α.
Figure 16 147
CHO cell human MIP-1α is inactive.
Figure 17 150
LD78α and LD78β amino-acid sequences including signal peptide.
Figure 18 155
The -4 amino-terminal truncated non-aggregating, bacterial recombinant form of human MIP-1α (M1) is active.
Figure 19 155
The activity of M1 on the KGM assay is reversible by a specific anti-human MIP-1α antibody.
Figure 20 156
Electrospray mass spectroscopy of purified COS7 MIP-1α.
Figure 21 158
Does proteolytic cleavage activate bacterial recombinant MIP-1α.
Figure 22 160
Proteolytic processing by interleukin-1β converting enzyme.
Figure 23 163
Reverse phase chromatography (RPC) run of GI MIP-1α.

Figure 24 164
Analysis of the GI MIP-1α reverse phase column fractions on the KGM assay.
Figure 25 165
Silver stain of the reverse phase column purified fractions 15 to 31.
Figure 26 166
The activities of the reverse phase purified fractions 19, 20 and 21.
Figure 27 167
The activities of the fractions 19, 20 and 21 are reversible by a specific polyclonal antibody to MIP-1α.
Figure 28 168
The activity of fraction 19 is not enhanced by the addition of bacterial recombinant MIP-1α.
Figure 29 172
Western blot of the MIP-1α mutants.
Figure 30 173
KGM assays of COS 7 murine MIP-1α mutants.
Figure 31 174
CFU-A assays of amino-terminally truncated mutants.
Figure 32 175
KGM assays of MIP-1α mutants derived in *Saccharomyces cervisiae.*
Figure 33 176
Dose-response activity of the -6 mutant in the KGM assay.

Abstract

Chemokines display a wide range of diverse functions and MIP-1α is no exception. It is not only a potent inflammatory molecule but also an inhibitor of primitive haemopoietic cell proliferation. The studies described in this thesis focus on the activity of MIP-1α on keratinocyte proliferation. These experiments have shown that a pure form of MIP-1α, derived from COS 7 cells by transient transfection, inhibits the proliferation of clonogenic human epidermal keratinocytes *in vitro*. This activity is fully reversible by specific anti-MIP-1α polyclonal antibodies. Despite this, the pure bacterial recombinant preparation of MIP-1α is largely inactive. In order to investigate this discrepancy, studies analysing the difference between these two MIP-1α preparations have been carried out. These studies suggest that the activation of the keratinocyte inhibitory function of MIP-1α results through a subtle structural modification which may involve the truncation of the amino-terminal amino acids. The potential role of MIP-1α in epithelial tissues *in vivo* is also discussed.

ABBREVIATIONS.

MIP	Macrophage inflammatory protein
5-FU	5-fluorouracil
ab	Antibody
AcSDKP	Tetrapeptide Inhibitor (acetyl-N-Serine-aspartate-lysine-proline)
AOP	Aminooxypentane
b	Bacterial
B1	Non-blue sepharose binding fraction
B2	Blue sepharose binding fraction
BB10010	MIP-1alpha non-aggregating mutant
BBT	British biotechnology
beta-TG	Beta-thromboglobulin
BFU-E	Burst forming unit erythroid
bMIP-1alpha	Bacterial derived MIP-1alpha
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
BUDR	Bromodeoxyuridine
C	Single cysteine chemokine family
CAFC	Cobblestone area forming cell assay
C-C	Beta chemokine family
CCE	Counterflow centrifugal elutriation
CCR	Beta chemokine family receptor
CFU	Colony forming unit
CFU-E	Erythroid colony forming units
CFU-GEMM	Granulocyte/erythroid/monocyte/macrophage Colony Forming Unit
CFU-GM	Granulocyte/monocyte colony forming unit
CFU-MK	Megakaryocyte colony-forming units
CHEF	Chinese hamster embryo fibroblast cell line
CHO	Chinese hamster ovary
c-kit	Stem cell factor receptor
c-mpl	Thrombopoietin receptor
CMV	Cytomegalovirus
COOH	Carboxy terminus
COS-	Supernatant derived from COS 7 cells
COS 7	SV40 transformed African Green Monkey kidney cell line
COS 7 MIP-1alpha	COS 7 cell derived MIP-1alpha
COS H1	Non heparin-binding COS 7 cell supernatant derived proteins
COS H2	Heparin binding COS 7 cell supernatant derived proteins
COS+	Supernatant derived from incubation of bmip-1alpha with COS 7 cells
CSF	Haematopoietic colony stimulating factor
CTAP-III	Connective tissue activating peptide-iii
CX3C	Chemokine family with 3 amino-acids separating first two cysteines
CXC	Alpha chemokine family
CXCR	Alpha chemokine family receptor
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor

EGF-R	Epidermal growth factor receptors
ELISA	Enzyme linked immunosorbent assay
EMS	Electrospray mass spectroscopy
EMU	Epidermal-melanin unit
EPO	Erythropoietin
EPP	Epidermal pentapeptide
EPU	Epidermal proliferative unit
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	FGF receptor
FPLC	Fast performance liquid chromatography
gamma-IP10	Gene inducible by gamma interferon
GCP	Granulocyte chemotactic peptide
G-CSF	Granulocyte colony stimulating factor
GI	Genetics institute, Cambridge, Mass., USA.
GI MIP-1alpha	Genetics Institute derived COS 7 MIP-1alpha
GM-CSF	Granulocyte macrophage colony stimulating factor
Gro	Autocrine growth factor
H&E	Haematoxylin and eosin
H1	Non-heparin binding fraction
H2	Heparin binding fraction
HB-EGF	Heparin-binding EGF-like Growth Factor
HEK	Human epidermal keratinocyte
Hepmut	Non-heparin binding MIP-1alpha mutant
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
HPP-CFC	High proliferative potential colony forming cell
huMIP-1alpha	Human MIP-1alpha
ICAM	Intercellular adhesion molecule
ICE	Interleukin 1-beta converting enzyme
IFN	Interferon
IL	Interleukin
IL-8R	Interleukin-8 receptor
IP3	Inositol triphosphate
K	Keratin
KGF	Keratinocyte growth factor
KGFR	KGF receptor
KGM	Keratinocyte growth medium (serum free)
Lab MIP-1alpha	MIP-1alpha derived from COS 7 cells in this laboratory
LAP	Latency associated peptide
LA-PF4	Low affinity platelet factor 4
LD78	Human MIP-1alpha
Lin	Lineage
LPS	Lipopolysaccharide
LTBMC	Long term bone marrow culture
LTBMC-IC	Long-term bone marrow culture initiating cells
LTRC	Long-term repopulating cell
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage/monocyte colony stimulating factor
MGSA	Melanoma growth stimulatory activity
MPIF	Myeloid progenitor inhibitory factor

Mr	Molecular weight
mRNA	Messenger RNA
mu	Murine
muMIP-1alpha	Murine MIP-1alpha
NAP	Neutrophil activating peptide
NH2	Amino terminus
P1/ P2/ P3/ P4	PCR primers
PAGE	Polyacrylamide gel electrophoresis
PBP	Platelet basic protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
pEEDCK	Inhibitory pentapeptide
PF-4	Platelet factor 4
PHSC	Pluripotential haematopoietic stem cells
PLC	Phospholipase C
PM	Non-aggregating MIP-1alpha mutants
pMTx	Parental pMTx plasmid
pMTx.MIP-1alpha	plasmid containing MIP-1alpha cDNA
Rantes	Regulated on activation normal T-cell expressed and secreted
Rb	Retinoblastoma protein
RPC	Reverse phase chromatography
RT-PCR	Reverse-transcriptase polymerase chain reaction
Sca	Stem cell antigen
SCF	Stem cell factor
SDF	Stromal derived factor
SDS	Sodium dodecyl sulphate
SLM	Special liquid medium
TCA	T-cell activation
TFA	Tri-fluoro acetic acid
TGF	Transforming growth factor
Thy	Thymocyte
TNF	Tumour necrosis factor
Wt	Wildtype

Statistical Analyses.

Paired t-tests have been used to test the difference between two sets of variables. The test has assessed the null hypothesis stating that the difference between the variables is zero. The significance levels have been quoted as p values, and were obtained using Microsoft Excel® for Windows 95. The error bars quoted in all the result figures give an estimate of the standard error of the mean of all the results obtained for that experiment.

Corrections.

The figures summarise the results of at least three experiments. MIP-1α concentrations were estimated using silver staining and Western blotting, and compared with known quantities of bacterial MIP-1α obtained from R&D systems. Since our results have suggested that the difference in the potencies of wildtype bacterial and COS 7 MIP-1α is at least several hundred fold, we anticipate that any potential minor inaccuracies in MIP-1α concentration estimation would be unable to mask the inhibitory potential of the active species.

1. Introduction

Tissues which undergo constant regeneration have developed a complex and carefully controlled system of replication and differentiation. The maintenance and regulation of these tissues is ultimately controlled at the level of the stem-cell. Stem cells are the most primitive cells in these populations and comprise only a small subpopulation of the tissue. Stem cells have been described as cells capable of extensive self-maintenance, being able to maintain their proliferative self-renewal potential for at least one natural life-span of the organism (Lajtha 1979). A stem cell also has the potential to produce differentiated progeny which are called progenitor cells. These cells have a limited mitotic capacity, which is why they are also sometimes termed transit amplifying cells. These progenitor cells can also differentiate into mature, fully functional, terminally differentiated cells.

All continually renewing tissues by definition contain stem cells and are responsible for maintaining the steady-state tissue population. However not all mammalian tissues require this maintenance since the rate of proliferation of the cells of a particular tissue depends largely on the rate of turnover of the cells in that tissue. Leblond (1964) classified adult tissues as falling into three main types. Tissues with static cell populations and no cell division such as muscle and the nervous system; tissues such as liver with little cell division normally, but with the potential, when required, to self renew; and tissues such as the haemopoietic system and stratified squamous epithelia which are normally characterised by rapid and continuous cell turnover. Here terminally differentiated cells are being continually lost and replaced by an intricate and highly regulated proliferative process.

1.1. Haematopoiesis.

Most early studies of stem cells involved the haemopoietic system. The haemopoietic system is a complex cellular system consisting of 8 to 10 mature cell types and an overlying hierarchy of more immature cells which maintain the required cellularity of the system through proliferation, commitment and differentiation. All eight mature blood cell types ultimately derive from a single pluripotent stem cell which differentiates through committed lineage-restricted progenitors to phenotypically and functionally distinct mature cell types. These mature cells have a limited life-span and therefore haemopoiesis is required constantly even under normal physiological conditions. This process is regulated with

precision, since circulating levels of mature cells are maintained within narrow limits of variation. However cell production can be altered rapidly in response to increased or decreased demand (Metcalf 1984).

Adult mammalian haemopoiesis occurs primarily in the bone marrow and has classically been viewed as being pyramidal in shape (Graham and Wright 1997) with three overlapping compartments comprising probably around 0.1% stem cells, approximately 3% progenitor cells, the remaining cells being differentiating end cells (Metcalf 1984). These terminally differentiated cells have remarkably diverse functions ranging from specific host defence by B and T lymphocytes to oxygen distribution by erythrocytes.

1.1.1. Stem cells

Historically the most conclusive evidence for the existence of stem cells first came from experiments in which grafting of haemopoietic tissue from normal mice into recipient mice, previously irradiated with lethal doses of radiotherapy, completely re-populated the recipient's bone marrow. Ford et al (1956) were the first to show that transfused bone marrow cells would rescue mice whose haematopoietic system was completely ablated by X-ray irradiation. This led to the development of a clonogenic stem cell assay by Till and McCulloch (1961), who showed that bone marrow grafting following myeloablative doses of radiotherapy produced splenic nodules containing cells of both myeloid and erythroid lineage, each nodule apparently being the offspring of a single bone marrow cell. Moreover cells from these splenic colonies, when pooled and injected into a second lethally irradiated mouse, were capable of both generating further splenic colonies and successfully rescuing the mouse from bone marrow failure. They termed the cells in these nodules spleen colony forming units or CFU-S. Other studies were able to show that these spleen colonies were clonal (Wu 1967). When bone marrow with a radiation induced chromosomal abnormality was transplanted, the chromosomal marker was present in all the CFU-S cells. This suggested that the CFU-S represented a marker for mouse pluripotential haematopoietic stem cells (PHSC). The individual CFU-S cells appeared to have extensive proliferative capacity as demonstrated by the fact that the macroscopic spleen colonies frequently contained more than a million cells (Siminovitch 1963). The CFU-S cell therefore appeared to be a good candidate for the PHSC.

It is however now well recognised that the CFU-S assay does not recognise one homogeneous cell type, but a heterogeneous population with respect to proliferation and differentiation capacity, self-renewal ability, and cell-cycle status depending on when the CFU-S assay is scored (Magli et al 1982). The first CFU-S colonies to appear arise on the spleen surface usually at days 7-8 post-transplantation and consist mainly cells of one haemopoietic lineage (usually erythroid), with little self-renewal potential. These colonies are destined to disappear within three days (Magli 1982). At day 12 post-transplant however new colonies become evident as the day 7 colonies start to regress. Studies of these CFU-S colonies involving serial transplantation have shown that these day 12 colonies have a much higher self renewal potential than day 7 CFU-S (Baines 1983). Other studies have shown that the day 7 CFU-S are very sensitive to cycle-specific cytotoxic drugs, whereas the day 12 CFU-S cells are relatively refractory suggesting that the day 7 CFU-S cells are largely proliferative while the day 12 CFU-S are mainly quiescent (Hodgson 1979).

Although the origin of the day 12 CFU-S is still a matter of debate, it is clear that the CFU-S assay recognises a heterogeneous stem cell population. These findings led investigators to suggest that the stem cell compartment is organised in a hierarchical structure, with young stem cells that have undergone a limited number of divisions having extensive self-renewal capacity but a limited tendency to differentiate; while on the other hand older stem cells, that is cells that have undergone a relatively large number of cell-divisions, have a limited self-renewal capacity and a greater tendency to differentiate. This hypothesis is termed the 'Generation Age Hypothesis' (Rosendaal 1979).

The CFU-S, in particular day 12 CFU-S, exhibits many of the characteristics of the PHSC with extensive self-renewal potential, extensive proliferative potential and the ability to generate multiple haemopoietic lineages. Moreover most approaches that enrich for PHSC also enrich for day 12 CFU-S, usually to a much greater degree than other assayable haemopoietic progenitors (Visser 1984; Spangrude 1988). However it is now clear that the CFU-S is not the earliest pluripotential haematopoietic stem cell. There is in fact little evidence to suggest that the CFU-S are functional progenitors of the lymphoid system, although clonal erythroid, granulocytic, monocytic and megakaryocytic cells are found in a CFU-S colony (Abramson 1977). It is also clear that the frequency of PHSC in mouse bone marrow is considerably less than the frequency of the day 12 CFU-S cells (Jones 1987).

There is also little correlation between the ability of bone marrow to bring about the long term repopulation of irradiated mice and its CFU-S content (Jones 1989). In serial bone marrow transplantation experiments, which are used to study the self renewal capacity of haematopoietic progenitors, there is little correlation between CFU-S content and long-term marrow repopulating ability. In serially transplanted marrow grafts that could not produce donor engraftment, CFU-S numbers, although reduced, were at levels that should have been sufficient to produce full haematopoietic reconstitution. Furthermore on the serial transplants that did not give donor marrow reconstitution there was an initial engraftment at 10-14 days post-transplant which disappeared by days 17-21. It appears that serial bone marrow transplantation dissociates two waves of engraftment after BMT. There is an initial but unsustained wave of haemopoiesis and a later sustained wave which results in long-term reconstitution. The CFU-S (both day 7 and day 12) appears to be responsible for the early unsustained engraftment while the PHSC or pre-CFU-S is responsible for the later sustained engraftment. The CFU-S is therefore now considered to be a primitive myeloid stem cell, while the more primitive pre-CFU-S cell, with both myeloid and lymphoid potential is the more primitive stem cell that gives rise to the CFU-S compartment (Hodgson 1979). The pre-CFU-S compartment can now be distinguished from day 12 CFU-S on the basis of rhodamine-123 dye retention (Ploemacher 1989). Marrow fractions displaying high rhodamine fluorescence have been shown to be day-12 CFU-S enriched while the rhodamine dull fraction are pre-CFU-S enriched. This was confirmed by Spangrude and Johnson (1990) who were also able to demonstrate that only the rhodamine dull cells were able to transfer long term haematopoietic reconstitution to mice upon serial BMT. Direct evidence for the role of the CFU-S in early transient engraftment and for the pre-CFU-S cell was derived from studies using counterflow centrifugal elutriation (CCE) by Jones and his colleagues in 1990. CCE is a procedure that sorts cells on the basis of size and density using a constant centrifuge rotor speed, marrow cells being selected out by changing the rate of medium flow. The fraction containing the largest cells was mainly enriched for CFU-GM (granulocyte/monocyte colony forming unit) and both day 7 and day 12 CFU-S. The intermediate fraction was enriched for both day 7 and day 12 CFU-S but depleted of CFU-GM, while the fraction containing the smallest cells contained cells that were mainly morphologically identified as lymphocytes and depleted of CFU-S. In order to study the

nature of each CCE fraction male marrow cells were elutriated and then each fraction was transplanted into female syngeneic mice. The origin of the transplanted cells was then determined by southern blotting with a Y-chromosome specific probe. The large cell fraction was found to give rise only to early transient engraftment despite the presence of large numbers of CFU-S and CFU-GM. Therefore this large cell fraction is relatively depleted of PHSC although it is enriched for committed progenitors. The transplantation of the small cell fraction did not rescue mice from lethal aplasia. However when female lethally irradiated mice were transplanted with a female large cell fraction and a male small cell fraction, the mice survived. These mice had an early wave of transient engrafters derived from the female large cell fraction. However at two months following transplantation all the mice had male multilineage haemopoietic cells. Therefore the small cell fraction which was virtually depleted of CFU-S and committed progentors and could not rescue mice from lethal aplasia contained the PHSC. The CFU-S cell therefore gives rise to the transiently engrafting cells but does not bring about long-term bone marrow repopulation which arises from the pre-CFU-S. In long-term bone marrow cultures the pre-CFU-S is also the cell that results in the long-term maintenance of the system. Therefore the pre-CFU-S is also termed the long-term repopulating cell or LTRC.

The CFU-S assay is therefore a useful measure of transiently engrafting stem cells, although it does not give an accurate quantitative measurement of the most primitive stem cell numbers. However it remains useful as a stem cell assay, although it is a difficult assay to perform and requires the use of large numbers of mice. In view of this a number of *in vitro* assays have been established and are usually preferred. As with the CFU-S assay, these assays also measure a heterogeneous cell population. These *in-vitro* assay systems include the cobblestone area forming cell assay (CAFC) (Ploemacher 1989), the CFU-A assay (Pragnell 1988; Lorimore et al 1990), the primitive haematopoietic colony forming cell with high proliferative potential assay (HPP-CFC) (Bradley and Hodgson 1979), blast cell colony forming assay (Suda 1983) and long term bone marrow cultures (Dexter 1977). These assays are largely dependent on the culture of bone marrow derived cells in the presence of the necessary growth factors, and are scored as a measure of the colony forming ability of these cells. These *in-vitro* assays have now been extensively used to investigate the stem cells more primitive to the CFU-S.

Since the pre-CFU-S stem cells have been shown to be resistant to the cycle-specific cytotoxic effects of 5-fluorouracil (5-FU) they are likely to be slow cycling cells (Lerner et al 1990). These cells however do enter the cell-cycle some days after 5-FU treatment at which time they are sensitive to this drug's cytotoxicity. Under normal conditions therefore only a small proportion of these long-term repopulating stem cells appear to be in active cell cycle at any one time, the remaining stem cells being in the quiescent or G_0 phase of the cell cycle (Burgess and Nicola 1983). However large numbers of mature cells are required to maintain normal haemopoiesis, which has a turnover of hundreds of billions of cells per day in an adult human including 200 billion erythrocytes and 70 billion neutrophils. This is possible because the progenitor daughter cell that results from the division of the stem cell, can through its proliferative capacity, result in quite extensive amplification. For example 5-6 divisions during the transit of these progenitors results in a 32-64 fold amplification, while as little as 10 divisions results in a 1024 fold amplification. Therefore the long-term repopulating cells or pre-CFU-S can under steady-state conditions be largely out of cycle. However it appears that under conditions of insult or stress to the system, for example by cytotoxic chemotherapy, these quiescent cells can be triggered into the cell cycle. This leads to 'more mature' actively cycling stem cells that can then replenish the system through their self-renewal and differentiation into progenitor cells that can proliferate extensively and through the process of differentiation result in mature terminally differentiated cells. Once the system is restored to its normal state, these cells appear to return to the quiescent phase (Becker et al 1965). This suggests that the stem cell and the progenitor cell are under both positive and negative proliferative regulation. Elucidating these control mechanisms is important for our understanding of normal stem cell regulation and may lead to an appreciation of the involvement of these factors in proliferative disease (Graham and Pragnell, 1991b). This will be discussed in the following sections.

1.1.2. Progenitor cells.

Stem cells must maintain their numbers through the lifespan of the individual while also generating cells that can undergo proliferation and differentiation. To maintain a constant pool of stem cells, one of two alternative processes might occur. First, for every stem cell that divides to yield two daughter stem cells by what is described as symmetric division, another

stem cell divides to yield two more differentiated, non-stem cells or progenitor cells whose purpose will be to populate the precursor pool. Alternatively, each stem cell division may actually be an asymmetric division. In this scenario one daughter cell from a stem cell division would leave the stem cell pool to become a committed progenitor, while the other daughter cell immediately returns to G_0 and replaces the parent stem cell in the stem cell pool. Progenitor cells differ from stem cells in that they have an altered proliferation and differentiation program, with a lower self-renewal capacity and a greater tendency to differentiate. These cells are recognised by their ability to form colonies containing one or more blood cell types in *in vitro* assays which usually are made up of semi-solid tissue culture medium containing agar or methylcellulose and the appropriate growth factors. Originally, cells from blood forming tissues were cultured with "feeder layers" containing other cell types such as normal embryo fibroblasts (Sachs 1992). These were chosen as possible candidates for the cells which can produce the regulatory molecules required for blood cell formation. This work eventually led to the discovery of clonogenic cells capable of giving rise to colonies containing macrophages, granulocytes or both in various stages of differentiation. Colony assays, similar in principle to this, have now been developed for many of the other identified haemopoietic lineages (Metcalf 1984). The mature cell types present in the colonies formed in each of these assay systems have been used to name each of the corresponding progenitor cell types from which they were derived. For example, cells giving rise to colonies containing granulocytes and macrophages are called CFU-GM or colony forming unit granulocyte-macrophage. Cells that give rise to red cells have been termed erythroid colony forming units or CFU-E, while the cells that give rise to megakaryocytes are termed the megakaryocyte colony-forming units or CFU-MK. These assays have led to the discovery and then the cloning of soluble factors which may be required for the proliferation, differentiation and survival of these progenitor cells under *in vitro* conditions.

1.1.3. Mature Terminally Differentiated Cells.

Following several divisions and lineage commitment decisions, a multilineage progenitor cell gives rise to several progeny, each of which is committed to a single lineage. These cells are the terminally differentiated progeny of the stem and progenitor cells and exhibit a remarkable range of functions ranging from non specific and specific host defence by white

blood cells, the control of haemostasis by platelets and the carriage of oxygen around the body by red blood cells. More than 8 major cell types are currently recognised. The functions of each of these cell types will not be discussed in detail in this text unless relevant.

1.2. The Regulation of Haemopoiesis.

Haemopoiesis therefore involves a highly complex series of cellular events in which a small population of stem cells continually generates large populations of maturing cells in a number of different lineages. Under normal conditions, this process, which involves diverse proliferative and differentiative events, appears to be precisely regulated. From the complexity of this process one would predict that the regulatory mechanisms involved in haemopoiesis are probably intricate and diverse. It is therefore not surprising that a large number of both positive and negative regulatory molecules have been identified and characterised. These include a number of the interleukins (IL-), erythropoietin, the haematopoietic colony stimulating factors (CSFs), stem cell factor (also known as kit ligand), thrombopoietin, transforming growth factor-β (TGF-β) and MIP-1α as well as various other cytokines such as the interferons and TNF-α .

1.2.1. Regulation of Haemopoiesis: The Stroma.

The bone marrow stroma is also thought to play a major role in the maintenance and regulation of haemopoiesis. Evidence obtained primarily from *in vitro* long term bone marrow cultures have shown that the primitive haematopoietic cells in this system are tightly bound to stromal cells while more differentiated cells are found as non-adherent cells (Coulombel et al 1983). Further studies suggest that a similar relationship occurs *in vivo* (Funk et al 1994). Stromal cells are thought to provide niches for the stem cells and can secrete some of the factors described below that can direct stem cell proliferation and differentiation (Muller-Sieburg and Deryugina 1995). Also while long-term bone marrow cultures are usually unable to maintain the long-term repopulating cell, recent studies have shown that rare stromal cells are able to maintain these cells (Wineman et al 1996). The mechanism by which this is carried out is not as yet clear but may involve an extracellular matrix or adhesion molecule, or a novel cytokine (Verfaillie et al 1993).

1.2.2. Regulation of Haemopoiesis: Growth Factors.

Growth factors are involved in the regulation of haemopoietic cell proliferation, differentiation and apoptosis.

1.2.2.1. Proliferation.

A considerable number of growth factors have now been characterised. Some of these cytokines are lineage specific and support the proliferation and the maturation of committed progenitors. This will be discussed in greater detail below. Examples include erythropoietin and thrombopoietin which are physiological regulators of erythropoiesis and megakaryocytopoiesis respectively, and M-CSF which is thought to be relatively specific to the macrophage/monocyte lineage.

However the larger proportion of these molecules are not lineage specific and can support the proliferation of multilineage progenitors. For example GM-CSF stimulates the proliferation of multipotential progenitors (Metcalf et al 1980). GM-CSF shares the same beta subunit as the IL-3 receptor which partly explains their overlapping functions (Kitamura et al 1991). IL-3 can, like GM-CSF, stimulate the formation of multilineage colonies which can contain granulocytes, macrophages, eosinophils, mast cells, erythroid cells or megakaryocytes. The actions of IL-3 by itself appear to be retricted mainly to multipotential progenitors since studies have shown that as haematopoietic progenitors differentiate, they generally lose their responsiveness to this cytokine (Koike et al 1986).

A number of growth factors are able to synergize with IL-3 to increase the colony numbers in *in vitro* assays. These include IL-6 (Ikebuchi et al 1987), G-CSF (Ikebuchi et al 1988), IL-11 (Musashi et al 1991), stem cell factor or c-kit ligand (Tsuji et al 1991) and the ligand for FLT-3/FLK-2 (Hudak et al 1995). These molecules synergise with IL-3 to increase the proliferation of these primitive cells. It has been suggested that these factors may be functioning by inducing the long-term repopulating cell/stem cell into cycle (Ogawa 1993), however this remains difficult to prove.

1.2.2.2. Differentiation.

It remains unclear what overall physiological role these growth factors play in the regulation of haemopoiesis *in vivo*. *In vitro* data suggests that they may be involved in determining differentiation along certain lineages e.g. granulocyte colony stimulating factor (G-CSF) in the differentiation of neutrophils; erythropoietin (EPO) in the production of erythrocytes; thrombopoietin in the generation of platelets and macrophage colony stimulating factor (M-CSF) for the monocyte/macrophage lineage.

However there is also evidence to suggest that in the presence of the proper permissive growth factors, the choice that a multipotent progenitor makes in determining which lineages to select upon cell division is random (Ogawa M. 1993). For example in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), which supports the differentiation along granulocytic, monocytic, eosinophilic, erythroid and megakaryocytic lineages, primitive haemopoietic cells commit to lineage restriction in an apparently random or stochastic manner (Nakahata T. et al 1982). Daughter cells arising from the single division of a primitive haemopoietic cell were separated by micromanipulation and then each cell was permitted to divide and differentiate to form secondary blast cell colonies. In a large proportion of the separated pairs the cells arising from each doublet daughter cell were totally different, with no apparent pattern connecting the two lineage compositions. Therefore at least within the confines of this culture system, while the presence or absence of specific haematopoietic growth factors appears to be important as permissive or perhaps 'happiness factors', individual cellular decisions to proceed down one or other of the permissible lineages seem to be stochastic or random events.

1.2.2.3. Apoptosis.

In the studies just described the growth factor appeared to be necessary *in vitro* as a permissive or 'happiness factor' which allows the multilineage primitive cell to commit to one or more lineages. There is in fact now evidence to suggest that the role of these molecules may be the suppression of apoptosis (Williams 1990). These cells would otherwise die by programmed cell death when removed from the bone marrow and the stem cell micro-environment. Further evidence for this role comes from work on the interleukin-3 dependent haematopoietic multipotent cell line FDCP-Mix (Fairbairn et al 1993). FDCP-Mix cells are

dependent on IL-3 for their continued survival and self-renewal, and upon withdrawal of this factor die by apoptosis. However when these cells are stably transfected with the bcl-2 gene, which has been shown to inhibit the apoptotic death of several factor-dependent cell lines, they are able to survive in the absence of IL-3 both in the serum-containing and in serum-deprived conditions. This survival is accompanied by multilineage differentiation in the absence of growth factors suggesting that the presence of a growth factor is not obligatory for the differentiation of stem cells although they may influence the lineage choice of multipotent cells. Furthermore these bcl-2 transfected FDCP-mix cells were seen to be able to differentiate without proliferating in single-cell experiments. This data suggests that the lineage commitment of the haemopoietic stem cell may be intrinsically determined by the cell's genetic program and not depend on haematopoietic growth factors. The primary roles of some growth factors *in vivo* may therefore be to suppress apoptosis.

1.2.2.4 The *in Vivo* Evidence: Growth Factor Knockout Mice.

On the other hand however, studies involving genetically manipulated mice deficient in one or more growth factors suggest that the presence of these factors may still be important for the normal differentiation process *in vivo.* In fact G-CSF knockout mice have a severe neutropenia which is reversible on treatment with the growth factor (Lieschke 1994a). However these mice still have low levels of mature neutrophils suggesting that either other growth factors are able to partially compensate for the loss of G-CSF, or that no growth factors are required for basal levels of neutrophil differentiation. To investigate this, studies involving transgenic mice in which two or more growth factors are knocked out have been pursued. Mice deficient in both GM-CSF and M-CSF have been reported. These mice have defects common to the GM-CSF (Stanley et al 1994) and M-CSF knockout mice, with defects in osteoclast and macrophage formation (as seen with the op/op M-CSF knockout mouse) (Yoshida 1990) and alveolar proteinosis lung pathology (as seen in the GM-CSF knockout mouse) (Lieschke et al 1994b). Mice lacking both G-CSF and GM-CSF have also been generated. These mice have a greater degree of neutropenia than mice lacking G-CSF alone, suggesting that the additional loss of GM-CSF in G-CSF animals further impairs steady-state granulopoiesis (Seymour et al 1997).

Further evidence for the importance of haemopoietic growth factors in vivo come from the Steel (Sl) and white spotting (W) anaemic mice, which are now known to be deficient in stem cell factor (SCF) and c-kit, its receptor respectively (Huang et al 1990). These mice have similar phenotypic abnormalities including severe anaemia and a decreased activity of primitive haemopoietic stem cells (Matsui et al 1990). Also mice which are deficient in the thrombopoietin receptor c-mpl have very low platelet and megakaryocyte counts with the other lineages being normal, demonstrating the functional importance of this molecule in platelet production (Gurney 1994). These results indicate that these growth factors are important in the normal production of physiological levels of mature blood cells.

1.2.2.5 Functional Redundancy?

It is becoming increasingly apparent as new regulatory factors are identified that there is a substantial degree of overlap in the *in-vitro* activity of a number of these molecules, much more than would appear necessary. This suggests that these molecules exhibit a high degree of functional redundancy (Metcalf D. 1993). For example the development of granulocyte colonies can be stimulated by what appears to be the direct activity of G-CSF, GM-CSF, IL-3, SCF or IL-6. Likewise at least seven or eight factors can stimulate or potentiate the formation of megakaryocyte colonies of one type or another and a similar picture is seen for eosinophils and mast cells. As has already been described it has been shown that a number of these molecules with broadly similar activities eg IL-3, GM-CSF, IL-5 share common receptor subunits. However these molecules also have discrete functions. This does tend to suggest true redundancy, however it does not exclude different signalling pathways for each regulator which may reveal important differences in the function of these molecules.

1.2.3. Regulation of Haemopoiesis: Inhibitors.

A number of negative regulators have been identified and proposed to be physiological regulators of haemopoiesis. These include γ-interferon, the inhibitory tetrapeptide AcSDKP(acetyl-N-Serine-aspartate-lysine-proline), the inhibitory pentapeptide pEEDCK (pGlutamate-Glutamate-Aspartate-Cysteine-Lysine), Transforming growth factor-β and the chemokine Macrophage inflammatory protein-1α (MIP-1α). These inhibitory factors will be

discussed in turn in the following sections. However although these molecules can inhibit haemopoiesis *in vitro* and *in vivo*, the physiological requirement for negative regulators of haemopoietic stem/progenitor cells remains a matter for debate. It has been suggested that to maintain haemopoietic homeostasis the stem cell can be regulated by either positive or negative control and may not require both. Therefore up or down regulation of positive factors is argued to be able to give adequate bi-directional control. This would appear to eliminate the need for negative growth regulators. It has however been shown that antisense TGF-β sequence significantly increases the rate of proliferation of haematopoietic precursors (Hatzfeld et al 1991), as can antibodies to TGF-β in an *in vitro* stem cell assay, the long term bone marrow culture or LTBMC (Eaves et al 1991). This suggests that this molecule is actively involved, at least in this system, in keeping the primitive progenitors in a quiescent state. Also, antibodies to the tetrapeptide haemopoietic inhibitor AcSDKP, have been shown to result in a modest but significant increase in the cycling of haemopoietic progenitors in vivo (Frindel 1989). These observations suggest that these molecules may play some role in haemopoiesis.

It has also been postulated that during the regenerative phase after cytotoxic chemotherapy, when most stem cells are actively in cycle presumably in response to secreted growth factors (Harrison and Lerner 1991), these rapidly proliferating primitive cells can quickly be brought to quiescence. It has been suggested that an inhibitory molecule or molecules may be actively involved in this process since if this control was exerted by the downregulation of positive growth factors there would be a longer delay before the return to the steady-state proliferation rate. However a number of studies have shown that these cells can rapidly (within minutes) alter the function/expression of their receptors and this could control stem cell proliferation in the absence of a stem cell-inhibitor. The maintenance of the G_0/quiescent state by stem cell inhibitors may on the other hand be critical in the absence of the necessary "happiness" growth factors, since stem cells have been shown to undergo apoptosis in these conditions unless kept in G_0. There is therefore both direct and indirect evidence for the role of these negative regulators in the physiological control of primitive haemopoietic cell proliferation.

1.2.3.1. Tetrapeptide Inhibitor AcSDKP.

The tetrapeptide AcSDKP was first identified as a low molecular weight inhibitory activity from foetal calf bone marrow in 1977 (Frindel and Guigon). This inhibitory activity was able to inhibit the entry of murine bone marrow CFU-S into cycle after cytosine arabinoside treatment. Moreover this activity was shown to protect mice from the toxicity of high dose cycle-specific cytotoxic chemotherapy in the form of cytosine arabinoside The structure of the molecule was eventually established from amino-acid sequencing, mass spectroscopy and nuclear magnetic resonance (Lenfant et al 1989).

This tetrapeptide is now chemically synthesised and the synthetic molecule's activity has been confirmed both *in vitro* and *in vivo*. There is some evidence to suggest that it is able to inhibit the entry into DNA synthesis of CFU-S after chemotherapy/radiotherapy, the cell cycling of murine CFU-A (Carde et al 1995), and the high proliferative potential colony forming cell (HPP-CFC) induced by stimulators (Robinson et al 1993). Further studies on long-term bone marrow cultures have shown that tetrapeptide reversibly decreases the proportion of high proliferative potential erythroid and granulopoietic progenitors in S-phase without decreasing their number (Cashman et al 1994). *In vivo* studies suggest that AcSDKP is able to significantly protect the stem cell pool from the toxicity of single-agent cytosine arabinoside chemotherapy (Bogden et al 1998). This molecule is therefore being further investigated in clinical trials.

Other studies have shown that tetrapeptide is constitutively produced in mice and that it is also synthesised and released by bone marrow cells in long-term bone marrow cultures (Grillon et al 1990). This molecule may therefore play a part in maintaining the physiologically quiescent state of haemopoietic stem cells.

1.2.3.2. Pentapepide Inhibitor.

The pentapeptide pyroGlu-Glu-Asp-Cys-Lys (pEEDCK) was originally isolated from normal human white blood cells as an inhibitor of the *in vitro* proliferation of murine myeloid progenitors (Laerum and Paukovits 1984). This small molecule is now chemically synthesised and its inhibitory activity has been shown to be dose dependent. It is a potent inhibitor resulting in 50% inhibition of colony forming unit granulocyte/macrophage (CFU-GM) colony formation at 10^{-12} M. Furthermore at high concentrations of this pentapeptide its

inhibitory effect is no longer seen (Laerum and Paukovits 1984) since its activity follows a bell-shaped dose response. This molecule may also function *in vivo* since it has been shown to reduce the numbers of CFU-S and to protect the pre-CFU-S *in vivo* from the toxicity of cytotoxic chemotherapy (Paukovits et al 1993).

The presence of a thiol group in pEEDCK makes it highly oxidation sensitive. Oxidation of the cysteine thiol groups leads to the formation of a disulphide bridged homodimer $(pEEDCK)_2$ which is a powerful stimulator of haemopoiesis *in vitro* and *in vivo* (Laerum 1988). This dimer appears to be a potent stimulator of progenitor cells and also of LTC-IC in long-term bone marrow culture (Paukovits 1995). It is therefore possible that a redox equilibrium exists between the inhibitory pEEDCK and the stimulatory dimeric $(pEEDCK)_2$ in the regulation of haemopoiesis.

The mechanism of action of this pentapeptide molecule has not yet been defined. Interestingly pEEDCK sequences have been identified in the $G_{i\,\alpha 1}$ chain of G proteins near the major phosphorylation site (Amatruda et al 1991). The significance of this is uncertain.

1.2.3.3. Tumour necrosis factor- α.

Tumour necrosis factor-α (TNF-α) is a multifunctional cytokine produced mainly by activated macrophages, but also by lymphocytes and endothelial cells (Kelker et al 1985; Cuturi et al 1987). TNF-α displays a broad spectrum of activities. While it was originally shown to induce necrosis of tumours (Pennica et al 1984), it has also been shown to have an effect on several different cell types (Sugarman et al 1985; Vilcek et al 1986; Nawroth et al 1986). In haemopoiesis TNF-α has been shown to act as both a positive and a negative regulator. TNF-α either alone or in combination with interferon γ has been reported to inhibit *in vitro* haemopoietic colony formation by granulocytic and monocytic (Murase et al 1987), erythroid (Roodman et al 1987) and multipotential progenitors (Murphy et al 1988). There has however been considerable variability in the results seen in these studies. This may be partly explained by the nature of the stimulus used in the various assays (Moore 1991). For example studies have shown that TNF-α can easily inhibit clonogenic assays stimulated by G-CSF, while colonies stimulated by GM-CSF are much more resistant to inhibition.

In contrast other studies have shown that TNF-α can, paradoxically, increase the proliferative response of primitive haematopoietic cells to cytokines such as IL-3 and GM-CSF (Caux et al

1990) in short term assays (5-10 days). Further studies have however shown that in the same assays TNF-α is a reversible inhibitor of subsequent (after 10 days) late IL-3 dependent granulocytic progenitor proliferation and differentiation (Caux et al 1991), supporting on the other hand monocytic progenitor growth.

The mechanisms of action of TNF-α in this bidirectional control are not fully understood but may involve a direct effect or an indirect one by regulating the production in other cells of haematopoetic growth factors such as G-CSF (Koeffler et al 1987), GM-CSF (Munker et al 1986) and M-CSF (Oster et al 1987). TNF-α is also able to modulate the expression of a number of cell-surface cytokine receptors, including the receptors for GM-CSF (Elbaz et al 1991), M-CSF (Jacobsen et al 1992), G-CSF (Shieh et al 1991) and IL-3 (Elbaz et al 1991). This modulation may in part explain some of the divergent results seen with TNF-α in haemopoietic assays.

Further work has suggested that TNF-α may suppress haemopoiesis by inducing apoptosis (Selleri et al 1995). In fact TNF-α has been shown to upregulate Fas antigen (CD 95) in primitive CD 34+ haemopoietic cells (Maciejewski et al 1995). Fas antigen is a member of the TNF-α receptor family which when activated by its ligand results in the transduction of a signal for programmed cell death (Itoh et al 1991). Fas is not normally expressed in CD34+ cells, although it may be expressed in some differentiated haemopoietic cells. Since both TNF-α and interferon-γ markedly upregulate Fas expression in CD34+ cells, some of the inhibitory activities previously described with these molecules may not have been transient inhibition of proliferation but cell death by apoptosis.

1.2.3.4. Interferons.

The interferons (IFN) are a heterogeneous family with a broad spectrum of biological activities which include antiviral, antiproliferative, immunomodulating, and differentiative effects. They have been implicated in the pathogenesis of aplastic anaemia (Zoumbos et al 1985) which has resulted in considerable investigation into their effects on haematopoiesis. The interferons have been subdivided into three main classes: IFNα, IFNβ, and IFNγ. A number of studies have suggested that all the different sub-groups of interferon are able to inhibit the proliferation of pluripotent haematopoietic progenitors (Neumann et al 1982; Broxmeyer et al 1983; Rigby 1985). Although initial studies were performed with partially

purified material, further work using bacterial recombinant preparations and specific neutralising antibodies has mainly confirmed the initial findings. These studies have confirmed that the interferons can indeed inhibit CFU-GM, CFU-GEMM, BFU-E and CFU-E as well as CFU-Mk (megakaryocyte) assays (Carlo-stella et al 1991; Ganser et al 1987). On the other hand interferon- γ has also been shown to be able to potentiate the proliferation of immature haematopoietic progenitors (Caux et al 1992; Kawano et al 1991). In the presence of IL-3 and to a lesser extent GM-CSF, interferon- γ stimulates progenitor proliferation, while IFN-α and IFN-β are inhibitory. On the other hand in the presence of G-CSF IFN-γ has been shown to either suppress progenitor proliferation or have no effect on progenitor proliferation. This is not dissimilar to what has been seen with TNF-α and may also be mediated by the regulation of the expression of other cytokine receptors. However unlike TNF-α which inhibits late granulocytic progenitor development while potentiating monocytic cell generation (see above), IFN-γ has no such effect.

Interestingly recent studies using stromal cells that were retrovirally transduced to produce IFN-γ, have shown that in LTBMC the IFN-γ secreted by these stromal cells was far more potent than exogenously added IFN-γ (Selleri et al 1996) in inhibiting secondary colony formation. It has been suggested that this incongruity may be explained by the fact that this cytokine may require specific matrix molecules for efficient presentation to the target cell (Gordon et al 1987; Roberts et al 1988) or may be presented as membrane bound molecules (Toksoz et al 1992). The inhibition observed appeared to be mediated partly through cell-cycle arrest but also by the induction of apoptosis in a significant proportion of cells. It is now clear that IFN-γ, like TNF-α, may suppress haematopoiesis by inducing programmed cell death in both lineage committed and non-lineage committed CD34+ cells (Selleri et al 1995). This may be mediated through the upregulation of Fas antigen (Maciejewski et al 1995). IFN-γ also induces a transcription factor IFN-regulatory factor-1 (IRF-1) which has been shown to regulate the transcription of genes involved in apoptosis (including p53), cell-cycle progression, and DNA repair as well as cellular metabolism. Activation of these pathways may determine the cellular response to this cytokine, however this is not fully understood.

1.2.3.5. Transforming growth factor-β (TGF-β).

The TGF-β Superfamily

The TGF-β family of cytokines comprises a large number of factors with very diverse activities, for which the structural prototype is the protein that was first isolated from human platelets as an activity described as TGF-β (Assoain et al 1983). This was cloned from a human cDNA library (Derynck et al 1985) and later named TGF-β1 (Cheifetz et al 1987). Further work revealed that this molecule was prototypic of a superfamily of growth, differentiation and morphogenesis factors including the inhibins, activins, the bone morphogenetic proteins, mullerian inhibitory substance as well as TGF-β2 and TGF-β3 (reviewed by Massague 1990; Sporn and Roberts 1992). Members of this family are related by sequence homology, the most conserved feature being the presence and position of nine cysteine residues in the mature carboxy terminus. The distribution of TGF-β related factors is widespread in organisms ranging from the fruitfly to humans and their evolutionary conservation is unusually strict which suggests that these molecules must play a fundamental role in the normal physiology of these species.

a) The TGF-β Subfamily: TGF-β Structure and Activation.

The main TGF-β subfamily consists of at least five members all of which have extensive sequence homology: TGF-β1, TGF-β2, TGF-β3, TGF-β4 and TGF-β5. TGF-β1 is the structural prototype and is a 25kDa disulphide-linked homodimer comprising two identical chains of 112 amino-acids. Each chain is synthesised as the C-terminal domain of a 390 amino-acid precursor that has all the characteristics of a secretory polypeptide, having an amino-terminal signal sequence which is necessary for transport out of the endoplasmic reticulum, a pro-region and the C-terminal bioactive domain (Gentry et al 1988; Wakefield et al 1988). This inactive 105kDa complex can be cleaved to generate the active cytokine and the pro-region, an 80kDa fragment which is also known as the latency associated peptide or LAP. Further studies have shown that association of the mature TGF-β molecule with the pro-region LAP can inactivate this factor (Gentry et al 1988), and that LAP is a potent inhibitor of TGF-β1, 2 and 3 both *in vivo* and *in vitro* (Bottinger et al 1996). Activation can be mediated *in vitro* by exposure to extreme pHs (<4 or >9), or exposure to other chaotropic agents such as sodium dodecyl sulphate (Lyons et al 1988) as well as the proteases

cathepsin D and plasmin. *In vivo* activation may involve plasmin. The evidence for this comes from human apolipoprotein (a) gene transgenic mice, a species that normally lacks apolipoprotein (a). The activation of TGF-β is inhibited in these mice. This probably results via the inhibition of plasmin generation from plasminogen by apolipoprotein (a), which suggests that plasmin is an important activator of TGF-β in vivo (Grainger et al 1994). Furthermore it has been shown that TGF-β can downregulate the expression of plasminogen activator, while upregulating the expression of plasminogen activator inhibitor (Laiho et al 1986a and b), which suggests that this cytokine can control its own activation. Other work has revealed that thrombospondin, an extracellular matrix molecule is found complexed with TGF-β in the α-granules of platelets (Schultz-Cherry et al 1993). Thrombospondin is able to activate TGF-β by forming specific complexes with TGF-β latency associated peptide in a reaction that does not require plasmin. Furthermore thrombospondin is also able to upregulate TGF-β expression. However the in vivo significance of this remains uncertain (Schultz-Cherry et al 1994a and 1994b).

b) TGF-β- A Multifunctional Cytokine.

The TGF-βs are multifunctional with a broad spectrum of diverse functions including both positive and negative effects on a number of different developmental and mature cell systems. Many other cytokines have been shown to have a wide range of very different activities (Sporn and Roberts 1988), however few have been implicated in as many different cellular processes as TGF-β (Sporn and Roberts 1992).

TGF-β has been shown to induce angiogenesis (Roberts et al 1986), chondrogenesis (Seyedin et al 1985) and osteogenesis (Seyedin et al 1987). TGF-β has also been shown to regulate cellular differentiation and proliferation in a number of different cell types, either positively or negatively - depending on the cell-type and growth conditions involved. This molecule is also thought to be involved in wound repair and fibrosis and can regulate the synthesis and degradation of extracellular matrix components (Reviewed by Noble et al 1992).

c) TGF-β and Haemopoiesis.

TGF-β is constitutively expressed in both the bone marrow and the peripheral blood (Cluitmans et al 1995) and is known to be expressed by a number of haematopoietic cell types including platelets from which TGF-β1 was first identified (Assoian et al 1983), activated macrophages (Assoain et al 1987), B-lymphocytes (Kehrl et al 1986a) and T lymphocytes (Kehrl et al 1986b). TGF-β has a strong claim to be an important player in the physiologic regulation of early haemopoiesis. It has also been shown to have a central role as an immunological mediator. In fact TGF-β has been shown to be not only a potent neutrophil chemoattractant (Reibman et al 1990), but also a powerful anti-inflammatory cytokine. *In vitro* it is able to prevent macrophages carrying out the respiratory burst (Tsunawaki et al 1988), inhibit B-cell and T-cell proliferation, inhibit T-cell and natural killer cell immune function, inhibit B-cell immunoglobulin (IgG and IgM) synthesis, control the generation of a number of cytokines as well as regulate the expression of a number of cytokine receptors (Keller et al 1990; Ranges et al 1987; Rook et al 1986).

In vivo evidence for the role of TGF-β as an anti-inflammatory molecule was derived from transgenic mice that had both the alleles for the TGF-β1 gene knocked out (Shull et al 1992; Kulkarni et al 1993). These mice have no obvious phenotype at birth but develop a wasting syndrome in the first few weeks of life secondary to a widespread and severe multifocal autoimmune reaction. This can be interpreted to indicate that there is considerable redundancy in the expression of the various forms of TGF-β and that TGF-β1 is not essential for normal development, only becoming crucial later on in life. However this is not the case since further studies have shown that the absence of a phenotype is actually related to the transfer across the placenta to the foetus of maternal TGF-β1 (Letterio et al 1994). In fact these mice develop a number of histological abnormalities after the first week of life as their levels of maternal TGF-β1 fall. Furthermore TGF-β1 null mice bred from a TGF-β1 null female have severe cardiac defects and die perinatally, implicating TGF-β1 in cardiac development. The TGF-β1 null mice born to heterozygous females on the other hand develop normally to term. These results have confirmed that TGF-β1 is a main player in the regulation of the immune response. These knockout mice experience a severe, multifocal, mixed inflammatory cell infiltration associated with tissue necrosis in most organs including

the lungs, heart, liver, and gastrointestinal tract. This leads to organ failure and death. These mice appear to have a severe autoimmune disorder and may be a useful model for the study of auto-immune disease. However not all TGF-β null mice develop this syndrome, and it appears that this phenotype is mouse-strain specific.

TGF-β is also well recognised as a regulator of the proliferation of a number of cell types. Since TGF-β has also been shown to induce apoptosis (Jacobsen et al 1995), as with TNF-α and the interferons it may be difficult to distinguish between inhibition of proliferation and apoptosis when interpreting TGF-β inhibition data. However it is generally thought that TGF-β is a potent inhibitor of the proliferation of primitive multipotent haemopoietic progenitors. In fact the *in vivo* administration of TGF-β1 or TGF-β2 can protect mice from otherwise lethal doses of myelotoxic chemotherapy (Grzegorzewski et al 1994). *In vitro* TGF-β has also been shown to directly and reversibly inhibit the growth factor driven proliferation of most primitive haematopoietic progenitors (Keller et al 1990; Sitnicka et al 1996). Sing et al (1988) showed that TGF-β1 and TGF-β2 were both able to inhibit colony formation, in the presence of either GM-CSF or IL-3, by erythroid (BFU-E), multipotential (CFU-GEMM) and granulocyte-macrophage (CFU-GM) by 90-100% at concentrations of 400pM. TGF-β has also been detected in long-term bone marrow cultures, where it can inhibit the cycling of primitive progenitors (Cashman et al 1990). Interestingly it has also been shown that the addition of anti-TGF-β antibody or LAP to long-term bone marrow cultures results in the activation from quiescence into cycle and an increased proliferation rate of the primitive haemopoietic cells in this system suggesting that TGF-β must be involved in the regulation of proliferation of these cells in this culture system and also indicates that TGF-β is an important physiological haemopoietic negative regulator (Eaves et al 1991).

Paradoxically TGF-β has also been shown to be able to stimulate the proliferation of more mature lineage restricted progenitors (Keller et al 1991) This bidirectional activity has been seen with a number of the negative regulators including the inhibitory pentapeptide, TNF-α, interferon-γ and MIP-1α (see below).

TGF-β has also been implicated in the regulation of differentiation of a variety of haematopoietic cells with a similar bi-modal activity. TGF-β has been shown to directly inhibit the IL-3 induced expression of the differentiation marker Thy-1 in Thy-1 negative cells,

suggesting that this molecule may inhibit the differentiation of primitive haematopoietic cells (Keller et al 1990). Paradoxically again, TGF-β has been shown to induce the erythroid differentiation of burst-forming units-erythroid (BFU-E) cells (Krystal et al 1994). TGF-β has also been observed to promote IL-3 dependent basophil differentiation (Sillaber et al 1992), while inhibiting eosinophil differentiation *in vitro.* However the *in vivo* significance of these findings are not clear, since the conditions brought about by *in vitro* culture are far removed from the bone marrow micro-environment. Such results must therefore be interpreted cautiously. Furthermore under these *in vitro* conditions the processes of proliferation and differentiation overlap making the interpretation of this type of data difficult.

As has already been noted TGF-β has also been observed to induce apoptosis in a number of different cell types, including primitive murine haemopoietic progenitors (Jacobsen et al 1995). TGF-β has been shown to be able to counteract the haemopoietic growth factor induced survival of single cell cultures of primitive murine haemopoietic (Lin- Sca+) progenitors by inducing apoptosis. These primitive haemopoietic progenitors show an absolute requirement for cytokines (such as IL-3 or SCF) to survive *in vitro* since no colonies are derived when the addition of cytokines is delayed for 40 hours. In the presence of the relevant cytokine and TGF-β these cells apoptose. Furthermore antibodies to TGF-β enhance the survival of these Lin- Sca+ progenitors by neutralising endogenous TGF-β, both in the presence and in the absence of haemopoietic growth factors (Jacobsen et al 1995). Other studies have shown that TGF-β can also induce apoptosis in more differentiated haemopoietic cells including eosinophils (Alam et al 1994) and B-cells (Chaouchi et al 1995) as well as in primary hepatocytes (Sanchez et al 1996), and in a number of different cell lines. These results suggest that at least part of the inhibitory effects seen by this molecule in the past may have been related to programmed cell death. However a number of studies have convincingly demonstrated reversible inhibition with TGF-β in primitive haemopoietic progenitors (Sitnicka et al 1996). Overall therefore these results suggest that the reversibility of TGF-β inhibition may partly depend upon the presence or absence of other signals and the cell phenotype.

1.2.3.6. MIP-1α and the chemokines.

MIP-1α is a 69 amino- acid, 8kDa heparin-binding protein that was initially purified and characterised from the supernatant of a lipopolysaccharide stimulated macrophage cell line RAW 264.7 (Wolpe et al 1987). This protein is now well characterised as a reversible inhibitor of primitive haemopoietic cell proliferation (Graham et al 1990), and has been shown to be a member of a large and rapidly expanding family of *chemo*tactic cyto*kines* known as the chemokines to which the next section is dedicated.

1.3 The Chemokines.

The MIP-1α family of proteins which is also known as the chemokines now has more than 30 distinct members (Reviewed by Baggiolini et al 1997). Most of these molecules appear to be multifunctional, at least *in vitro*, with very diverse functions described. The first chemokine to be purified was platelet factor 4 (PF-4) (Handen and Cohen 1976), and novel molecules are still regularly being identified and characterised. Most of these molecules have been identified either through the investigation of a biological activity, or through molecular biological screening procedures designed to find cDNAs that are highly inducible (Zipfel et al 1989) or that encode secreted proteins (Tashiro et al 1993). The members of this family are both structurally and to some extent functionally related, possessing limited sequence homology and usually four positionally conserved cysteine residues arranged in either of two characteristic motifs (Reviewed by Miller and Krangel 1992). The presence or absence of an intervening amino-acid between the first two cysteines in these peptides divides the chemokine family into two main groups, the CXC and CC groups. Two other chemokine groups have also been described including the single-cysteine -C-/lymphotactin class (Kelner et al 1994; Dorner et al 1997), and the fractalkine CX3C class, which have 3 amino-acids separating the first two cysteines (Bazan et al 1997).

The CXC subfamily chemokines are generally described as neutrophil chemoattractants, while the CC subfamily members are generally thought to be monocyte chemoattractants. However this is at best a generalisation, since some of these molecules have overlapping activities. The genes for the CXC or α chemokines have been mainly mapped to a locus on human chromosome 4 (q12-21), while the genes for the C-C or β chemokine family are largely located on human chromosome 17 (q11-32). The chemotactic cytokine lymphotactin

only has three cysteines and has been classified separately into the C- subgroup of chemokines of which it is at present the only member (Kelner et al 1994; Dorner et al 1997). This molecule chemoattracts lymphocyte subsets but not neutrophils nor monocytes.

Fractalkine is a CX3C chemokine and is unlike all the other chemokine types. It can exist in two forms: either a membrane-anchored or a secreted 95KDa glycoprotein. The soluble CX3C chemokine has potent chemoattractant activity for T cells and monocytes. The cell-surface-bound protein, which is induced on activated primary endothelial cells, promotes strong adhesion of those leukocytes. The structure, biochemical features, tissue distribution and chromosomal localization of this CX3C chemokine all indicate that it represents a unique class of chemokine that may constitute part of the molecular control of leukocyte traffic at the endothelium (Bazan et al 1997).

1.3.1 The CXC chemokines (α-chemokines).

1.3.1.1 Platelet factor 4 (PF-4).

PF4 was the first chemokine to be characterised. It was first recognised as a platelet protein with anti-heparin activity (Deutsch et al 1955). The eventual purification of this activity from platelet α-granules led to the sequencing of a 7.8kDa 70 amino acid protein (Deuel et al 1977). Further work revealed that this cytokine is a weak chemoattractant for neutrophils and monocytes (Deuel et al 1981) as well as a more potent fibroblast chemoattractant (Senior et al 1983). In addition PF4 has been reported to promote the proliferation of fibroblasts as well as the synthesis of glycosaminoglycans by chondrocytes (Senior et al 1983). These findings suggested not only a possible link between the initiation of thrombosis and the induction of inflammation, but also a possible role for this molecule in wound healing. More recent investigation has shown that this chemokine is an inhibitor of angiogenesis. It has been shown to inhibit angiogenesis in chicken chorioallantoic membranes and to inhibit the proliferation of human umbilical endothelial cells (Maione et al 1990). Further work has shown that this chemokine can inhibit the proliferation of endothelial cells derived from a number of different sources by inhibiting the transition from G1/G0 to S phase, as well as inhibiting DNA synthesis of cells already in S phase (Gupta and Singh 1994). These experiments however required unusually high concentrations of PF4 for endothelial cell growth inhibition (micromolar range as compared to nanomolar concentrations for other

chemokine activities). However when a potent inhibitor of endothelial cell proliferation was purified from media conditioned by activated human peripheral blood leukocytes, the inhibitory activity was found to be due to an amino-terminally truncated form of PF-4, generated by peptide-bond cleavage between Thr-16 and Ser-17 (Gupta et al 1995). This processed form of PF4 inhibits at nanomolar concentrations suggesting that endogenous amino-terminal processing of this and perhaps other chemokines plays an important role in determining the biological activity and receptor binding of these cytokines.

1.3.1.2 Platelet Basic Protein (PBP).

In the purification of the antiheparin activity from platelet α-granules that led to the identification of platelet factor 4, another platelet secretory protein, initially termed β-thromboglobulin (β-TG) was recognised. When the complete primary structure of this protein was determined (Begg et al 1978), it was found that this molecule showed about 50% homology with that of platelet factor 4 as well as sharing the motif of cysteine residues. Additional studies identified two other platelet proteins known as Connective Tissue Activating Peptide-III (CTAP-III) and Low Affinity Platelet Factor 4 (LA-PF4), which on amino-terminal sequencing were found to be amino-terminally extended forms of β-thromboglobulin (Castor et al 1979; Rucinski et al 1979). Further work confirmed that proteolytic cleavage of LA-PF4 can generate β-TG. The precursor of all of these proteins was eventually characterised from thrombin-stimulated fresh human platelets (Holt et al 1986) and termed Platelet Basic protein (PBP). PBP can generate β-thromboglobulin by proteolytic cleavage with trypsin or plasmin. More recently another distinct PBP cleavage product has been characterised from LPS stimulated human mononuclear cells. This variant has been termed Neutrophil Activating Peptide-2 (NAP-2) since it has a nearly identical amino-terminus and similar bioactivity to the previously characterised protein NAP-1 which is now known as interleukin-8 (Walz et al 1989). Furthermore monocye conditioned medium has been shown to cleave purified CTAP-III into NAP-2 through monocyte derived proteases (Walz and Baggiolini 1990). Interestingly NAP-2 can also be generated from platelet derived CTAP-III by the proteases chymotrypsin and cathepsin G (Car et al 1991), suggesting that these proteases or other similar activities result in the formation of NAP-2 *in vivo*.

Platelet Basic Protein(PBP): SSTKGQTKRNLAKGKEESLDSDLYAELRCMCIK.....

LA-PF4: RNLAKGKEESLDSDLYAELRCMCIK.....

CTAP-III: NLAKGKEESLDSDLYAELRCMCIK.....

β-thromboglobulin(β-TG): GKEESLDSDLYAELRCMCIK.....

NAP-2: AELRCMCCIK.....

Interestingly the main PBP variant found in platelets is CTAP-III, with the parent molecule PBP making up approximately 10-30% of the total PBP antigen, β-TG has not been detected in unstimulated platelets. It is thought that additional proteolyis may occur following platelet activation, perhaps during degranulation. However it is not clear whether this proteolysis of PBP is regulated or stochastic. There is however evidence to suggest that the biological functions or potencies, or both, of these molecules can be regulated in this manner since although the precursor of all these molecules PBP, appears to be totally devoid of activity, its cleavage products have been described to have a number of biological functions. NAP-2 is a potent chemoattractant and activating factor for neutrophils (Walz et al 1989), while β-TG is a fibroblast chemoattractant (Senior et al 1983). Finally both CTAP-III and NAP-2 have been shown to be heparin/heparan sulphate degrading enzymes (Hoogewerf et al 1995), and a recent report indicates that NAP-2 can also inhibit the formation of growth factor induced megakaryocyte colony formation (Gewirtz et al 1995).

1.3.1.3 γIP-10

γIP-10 was the next CXC chemokine identified, as a gene induced by γ-interferon (IFN-γ) in monocytes, fibroblasts and endothelial cells (Luster et al 1985). It was found to display a significant homology to PF-4 and β-thromboglobulin, contain the same cysteine motif, and to map to human chromosome 4 (q21) near the PF-4 gene (Luster et al 1987). This chemokine has been shown to be expressed in IFN-γ stimulated keratinocytes, macrophages, fibroblasts and endothelial cells (Gattass et al 1994). High levels of γIP-10 are also constitutively expressed in lymphoid tissues, including the spleen, thymus and lymph nodes although normal unstimulated lymphocytes do not express this chemokine. However γIP-10 is expressed constitutively by thymic and splenic stromal cells, as well as activated T cells and thymocytes. γIP-10 has been shown to be a chemoattractant for monocytes and T-

lymphocytes, and to promote T cell adhesion to endothelial cells (Taub et al 1993). It has also been shown to have a potent in vivo anti-tumour effect that is T cell dependent (Luster et al 1993). Finally it has been reported that this chemokine, like PF-4 and probably by an identical mechanism, inhibits endothelial cell proliferation by a specific but non-calcium fluxing, cell-surface heparan sulphate binding site (Luster et al 1995).

1.3.1.4 Interleukin-8 (IL-8).

Interleukin 8 (IL-8), also known as Neutrophil Activating Peptide-1 (NAP-1), was initially described as a cDNA clone expressed in activated human lymphocytes that encoded a protein with a high level of homology to PF-4 and PBP (Schmid and Weissmann 1987). At the same time a monocyte-derived neutrophil activating peptide was purified and found to be identical to the protein encoded by this cDNA by a number of independent groups (Schroder et al 1987; Walz et al 1987 Yoshimura et al 1987). The cDNA was found to encode a polypeptide of 99 amino-acids, including a putative amino-terminal signal sequence. Furthermore sequence analysis of mature IL-8 revealed that this chemokine exists in multiple forms that differ in truncation at the amino-terminus. Interestingly it has been shown that the composition of IL-8 preparations depends on the cell source used to produce it (Van Damme 1989; Schroder 1990). The most common forms of IL-8 are 72- and 77-amino-acids long (Lindley et al 1988; Gimbrone et al 1989), although 79, 71, 70 and 69 amino-acid forms of the chemokine have also been described.

Interestingly it has been shown that while endothelial cells secrete primarily the 77 amino-acid form, T-cells and monocytes secrete primarily the 72 amino-acid form (Hebert et al 1990). These investigators also reported that thrombin can convert the 77 amino-acid form into the 72 amino-acid form. Furthermore a number of groups have shown that the 72 amino-acid protein is at least 10-fold more potent *in vitro* than the longer form (Hebert et al 1990; Nourshargh et al 1992; Clark-Lewis et al 1991), although when tested *in vivo* however both forms are equipotent suggesting that the longer form may be cleaved *in vivo* by amino-peptidases to yield the more active form (Nourshargh et al 1992). It is also interesting to note that LPS stimulated monocytes have also been shown to secrete the 70 and 69 amino-acid variants of IL-8 (Lindley et al 1988) which have been shown to be about 5-fold more potent than the 72 amino-acid form in a number of functional assays (Clark-Lewis et al 1991).

IL-8 (79aa): EGAVLPRSAKELRCQCIKTYS.....

Natural variants: AVLPRSAKELRCQCIKTYS.....

SAKELRCQCIKTYS.....

AKELRCQCIKTYS.....

KELRCQCIKTYS.....

ELRCQCIKTYS.....

A number of other cell-types have been shown to produce IL-8. Apart from the already mentioned monocytes, T-lymphocytes and endothelial cells, other sources of IL-8 include fibroblasts (Van Damme et al 1989; Schroder et al 1990), keratinocytes (Larsen et al 1989), hepatocytes (Thornton et al 1990), synovial chondrocytes (Brennan et al 1990), articular chondrocytes (Lotz et al 1992) and mesothelial cells (Goodman et al 1992). In general it would appear that the cells that produce IL-8 are involved in the complex physiological responses of inflammation, immunity and wound-healing. Furthermore the signals that induce IL-8 secretion are usually pro-inflammatory, and include cytokines like IL-1α, IL-1β and TNF-α and bacterial products such as endotoxin. This suggests that IL-8 is a pro-inflammatory cytokine involved in the inflammatory cytokine cascade. Indeed IL-8 has been shown to act primarily on neutrophils, being a potent neutrophil chemoattractant (Yoshimura et al 1987; Lindley et al 1988) as well as a powerful neutrophil activator (Walz et al 1987). IL-8 has been shown to induce neutrophil degranulation and the neutrophil respiratory burst, induce a transient rise in neutrophil cytosolic free calcium, as well as upregulate neutrophil adhesion molecules. It has been reported that this upregulation can trigger adhesion of neutrophils to endothelial cells, thereby regulating neutrophil transmigration across blood vessel walls (Huber et al 1991). Interestingly in IL-8 transgenic mice (which have raised serum levels of IL-8 and increased numbers of neutrophils in the circulation, lungs, liver and spleen) there is no evidence of neutrophil extravasation or tissue damage, and neutrophil migration in response to IL-8 is severely impaired suggesting that an IL-8 gradient is necessary for its chemotactic activity (Simonet et al 1994).

Other research has shown that IL-8, in contrast to PF-4, is a potent angiogenic factor when implanted into rat corneas and is capable of stimulating chemotaxis and proliferation of human umbilical vein endothelial cells (Koch et al 1992). IL-8 has also been shown to be

chemotactic for T-lymphocytes (Larsen et al 1989) and fibroblasts (Dunlevy and Couchman 1995) and a recent report has also shown that IL-8 can inhibit the formation of megakaryocyte colonies in vitro (Gewirtz et al 1995). Furthermore IL-8 has been shown to stimulate keratinocyte proliferation (Sticherling et al 1989), and its presence in psoriatic plaques has resulted in this chemokine being implicated in the pathogenesis of psoriasis (Gillitzer et al 1991).

The cDNAs for two human IL-8 receptors have been cloned so far: IL-8RA and IL-8RB (Holmes et al 1991; Murphy et al 1991). These two molecules are 78% homologous, however despite this they have different chemokine binding patterns. This will be discussed further in the following sections. However it is interesting to note at this point the knockout mouse phenotype obtained when both alleles of the mouse IL-8RA gene were knocked out (Cacalano et al 1994). Firstly as one would expect the neutrophils from these mice migrate poorly in chemotaxis assays *in vitro* in response to the IL-8RB chemokine agonists. Furthermore these mice show a severely suppressed neutrophil migration response to the peritoneal injection of a chemical irritant suggesting that the murine ligand for this putative IL-8 receptor is a major physiological determinant of neutrophil-mediated inflammation (Lee et al 1995). Surprisingly these knockout mice had a massive expansion of neutrophil and B-cell numbers in bone marrow, lymphoid tissue and peripheral blood. It has subsequently been shown that when these mice are raised in a germ-free environment, the blood neutrophil counts are not raised. This suggests that the defect in neutrophil chemotaxis results in an inability to eliminate infection which may result in the release of cytokines that stimulate neutrophil generation (Moore et al 1995). Interestingly however these mice do not appear to be more susceptible to spontaneous infection.

1.3.1.5 GRO α,β,γ.

There have been three different human gro genes identified, gro-α, gro-β and gro-γ. The CXC chemokine gro-α was first identified through a subtractive hybridisation approach, as an mRNA expressed by a tumorigenic chinese hamster embryo fibroblast cell line (CHEF) but not in a similar non-tumorigenic cell line (Anisowicz et al 1987). The human homolog was also characterised and the genes found to encode CXC chemokine proteins. Furthermore independent workers purified an autocrine growth factor, which they called Melanoma Growth

Stimulatory Activity (MGSA), from the conditioned media of a human melanoma cell line and found this to be identical to gro-α (Richmond et al 1988) and to map to the CXC chemokine locus on chromosome 4. Further work has shown that gro-α is secreted by endotoxin-treated human monocytes and is a neutrophil chemotactic and activating protein (Schroder et al 1990). Subsequently other cDNAs have been cloned that are highly homologous to gro-α and there are now at least three different gro-genes (Tekamp-Olsen et al 1990; Iida et al 1990; Haskill et al 1990). Gro-β is 90% homologous to gro-α and gro-γ is 86% homologous. These molecules have been shown to be expressed in a number of different cell types including fibroblasts, endothelial cells and keratinocytes. MGSA has in fact, like IL-8, been purified from the conditioned medium of cultured psoriatic epidermal keratinocytes as a neutrophil-activating activity (Schroder et al 1992). MGSA has also been shown to be expressed in the epidermis in the differentiated (suprabasal) and non-proliferating keratinocyte layers, although *the in-vivo* significance of this is uncertain (Nanney et al 1995). Like the other chemokines however it is clear that these molecules have a number of very divergent activities. Apart from being potent inflammatory mediators, they have also been shown to be regulators of cell proliferation and more recently regulators of collagen secretion by fibroblasts (Unemori et al 1993).

1.3.1.6 The Stromal Derived Factors-1 α and β.

The chemokine SDF-1 was initially cloned as a gene constitutively expressed in bone marrow stromal cells (Tashiro et al 1993). Unlike the other CXC chemokines the SDF-1 gene maps to a locus on human chromosome 10. Furthermore sequence homologies have shown that SDF-1 is related almost equally to both the CC and CXC families. SDF-1α and SDF-1β arise by differential splicing from a single gene, and differ in four carboxyl-terminal amino-acids that are present in SDF-1β but not in SDF-1α. SDF-1 has been shown to be a T- lymphocyte chemoattractant and is also active on monocytes but not neutrophils. It also stimulates pre-B cell proliferation (Nagasawa et al 1994), and knockout mice lacking this chemokine have been found to have a severe depletion of B-cell lymphopoiesis as well as a reduction in bone marrow myelopoiesis (Nagasawa et al 1996). This suggests that this molecule is critical for normal lymphopoiesis and myelopoiesis. SDF-1 has also been shown to be the ligand for the chemokine receptor Lestr/fusin which has now been designated CXCR4 (Oberlin et al 1996).

This receptor has been shown to function as a co-factor for the entry of T-Tropic HIV-1 virus into T cell lines. Moreover SDF-1 inhibits HIV-1 entry into these T-cell lines (Bleul et al 1996). SDF-1 has also been recently shown to be a chemotactic factor for CD34+ haemopoietic progenitor cells (Aiuti et al 1997).

1.3.1.7 Other CXC chemokines.

A number of other CXC chemokines have been identified from a variety of cell sources. These include Granulocyte chemotactic peptide-2 (GCP-2) (Proost et al 1993), MIG which was identified as a cDNA induced in monocytes by γIFN (Farber 1990) and ENA-78 (Walz et al 1991) which is a 78 amino-acid long, neutrophil activating protein secreted by epithelial cells As these molecules are characterised it is becoming increasingly clear that the CXC chemokine sub-family comprises a large number of multifunctional proteins, often with overlapping functions which suggests a degree of functional redundancy.

1.3.2 The C-C chemokines (β-chemokines).

The C-C subfamily comprises more than 20 members with different degrees of amino-acid sequence homology ranging from 25-70% (Baggiolini et al 1997). This group of chemokines maps to human chromosome 17 and includes the monocyte chemoattractant proteins MCP-1, MCP-2, MCP-3, MCP-4 as well as the macrophage inflammatory proteins MIP-1α and MIP-1β, Rantes (Regulated on Activation, Normal T-cell expressed, and secreted), Eotaxin, I-309 which is the human homolog of murine TCA-3; HCC-1, HC-14, C10 and the gene *fic*. These chemokines share the typical primary structure of a cleaved signal peptide of between 20-24 amino-acids and a mature secreted protein which is usually about 70-80 amino-acids long, as well as the four positionally conserved cysteines with no intervening amino-acid between the first two. Proteins of the C-C subfamily in general do not bind CXC receptors, and conversely the CXC chemokines do not bind CC receptors (Reviewed by Miller and Krangel 1993).

1.3.2.1 The Macrophage Inflammatory Proteins: MIP-1α and MIP-1β.

These chemokines were the first c-c chemokines identified and will be discussed in detail in the section 1.3.3.

1.3.2.2 The Monocyte Chemotactic Proteins: MCP-1, MCP-2, MCP-3, MCP-4.

MCP-1 is probably the best characterised CC chemokine. It was first cloned as a Platelet Derived Growth Factor (PDGF) induced gene in murine 3T3 fibroblasts and designated JE (Rollins et al 1988). Its amino-acid sequence was found to contain the characteristic CC family cysteine motif. Further independent work led to the characterisation of two very similar proteins with molecular weights of 13 and 15kDa from PHA stimulated human monocytes (Yoshimura et al 1989a) and apparently identical proteins from the conditioned media of a human glioma cell line (Yoshimura et al 1989b). These proteins were potent monocyte chemoattractants and had blocked amino-termini. When these two proteins were sequenced they were found to have identical amino-acid primary sequences (Robinson et al 1989) but different degrees of O-linked glycosylation; (Jiang et al 1990,1991). Since this protein was a potent chemoattractant for monocytes, it was termed Monocyte Chemotactic Protein-1 (MCP-1). Natural post-translationally modified variants of MCP-1 have been identified from

mononuclear cell supernatants, and this protein has been shown to be secreted as not only the wildtype molecule but also as amino-terminally truncated and carboxy-terminally processed variants with altered bioactivity (Proost et al 1998). MCP-1 has also been shown to be expressed in activated fibroblasts (Strieter et al 1989; Yoshimura et al 1990) and lymphocytes (Kaczmarek et al 1985), monocytes (Rollins et al 1989), keratinocytes (Barker et al 1990), stimulated endothelial cells (Strieter et al 1989) and stimulated vascular smooth muscle cells (Valente et al 1988). A wide variety of malignancies and tumour cell lines have also been observed to express MCP-1 (Reviewed by Miller and Krangel 1992), but the biological relevance of this is as yet undefined.

MCP-1 has been shown to have a wide variety of different immunological functions. Although MCP-1 is inactive on neutrophils, it is a potent monocyte chemoattractant. MCP-1 also chemoattracts CD4+ and CD8+ T-cells (Carr et al 1994; Loetsher et al 1994; Taub et al 1995a), basophils (Bischoff et al 1992), and Natural Killer cells (Maghazachi et al 1994). Furthermore MCP-1 is a potent monocyte activator inducing the respiratory burst, an increase in cytosolic free calcium and the increased expression of β2 integrins (Reviewed by Baggiolini and Dahinden 1994). It is also a potent activator of histamine release in basophils. However eosinophils do not respond to MCP-1.

MCP-1 is active at subnanomolar concentrations. Its activities often follow a bimodal dose-response with decreasing activity at higher concentrations beyond peak activity. Interestingly transgenic mice which constitutively overexpress high levels of MCP-1 protein have been shown to have no evidence of monocytic infiltrates or any other inflammatory response (Rutledge et al 1995). However these mice are more susceptible to infection by intracellular pathogens such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*. This suggests that high levels of MCP-1 do not result in an inflammatory resonse, perhaps because of receptor desensitisation or a loss of the chemoattractant gradient. MCP-1 has however been implicated in a number of disease processes as diverse as rheumatoid arthritis, atherosclerosis, pulmonary inflammatory diseases, psoriasis and multiple sclerosis (Koch et al 1992; Porreca et al 1996; Zhang et al 1994; Gillitzer et al 1993; Ransohoff et al 1993).

More recently a number of other C-C chemokines have been cloned that are highly homologous to MCP-1 both in sequence (>60% homology) and in activity (Proost et al 1996). These molecules have been designated MCP-2, MCP-3 and MCP-4 (Van Damme et

al 1992; Uguccioni et al 1996). As these proteins are characterised it is becoming clear that there are some differences in cellular specificities between these molecules. All the MCPs chemoattract monocytes and lymphocytes, however while MCP-1 is a potent basophil activator that does not attract eosinophils, MCP-2 is able to stimulate both basophils and eosinophils. Furthermore MCP-3 appears to have the broadest range of activities, acting on dendritic cells, natural killer cells, eosinophils and basophils as well as neutrophils (Proost et al 1996). MCP-4 appears to share the activites of MCP-3 and Eotaxin, with both of which it shares over 60% sequence homology.

1.3.2.3 Eotaxin.

Eotaxin is a C-C chemokine that appears to act primarily on eosinophils. It was initially characterised from an established guinea pig model of allergic inflammation as a potent eosinophil chemoattractant activity and found to be highly homologous to human MCP-1 (Jose et al 1994; Kitaura et al 1996). Murine and human homologs have been subsequently cloned (Ponath et al 1996a). It has been been shown to be constitutively expressed in a number of organs including the lungs, and is rapidly induced on allergen exposure (Rothenberg et al 1995a) in these same organs. Eotaxin is rapidly expressed by IFNγ activated endothelial cells (Rothenburg et al 1995b) and is also expressed by epihelial cells (Aguirre et al 1995). It has been shown to rapidly recruit eosinophils to its site of injection in vitro (Collins et al 1995). Eotaxin appears to be a highly specific eosinophil chemoattractant (Garciazepedia et al 1996) with no activity on monocytes or neutrophils. The eotaxin receptor CCR3 cloned from human eosinophils appears to primarily bind Eotaxin and MCP-4, binding MCP-3 and Rantes with much weaker affinity (Uguccioni et al 1996; Daugherty et al 1996; Ponath et al 1996). Eotaxin appears to be a highly specific chemoattractant for eosinophils and may play an important role in a number of eosinophil mediated inflammatory disorders such as asthma. The *in vivo* administation of Eotaxin has been shown to induce pulmonary eosinophilia, suggesting that this molecule may in fact be involved in the pathogenesis of inflammatory respiratory disorders (Rothenberg et al 1996). More recent work has shown that Eotaxin knockout mice that have both alleles of the gene knocked out, have defective early eosinophil recruitment after antigen challenge (Rothenberg et al 1997). The late recruitment

of eosinophils remains unaltered, however these knockout mice do appear to have altered constitutive eosinophil levels.

1.3.2.4 Rantes.

Rantes (**r**egulated on **a**ctivation **n**ormal **T**-cell **e**xpressed and **s**ecreted) was originally identified as an activated T-cell expressed gene that directs the synthesis of an 8kDa protein (Schall et al 1988). Further studies have confirmed Rantes to be a member of the chemokine family with considerable sequence homology to MIP-1α. Mapping studies have also shown that like the other C-C chemokines the human Rantes gene lies on chromosome 17q (Donlon et al 1990). Further work has confirmed that like MCP-1, Rantes is a potent chemoattractant for monocytes and specific T-lymphocyte subsets, but not neutrophils (Schall et al 1990). Rantes is also a potent eosinophil and basophil chemoattractant and activator (Kameyoshi et al 1992; Rot et al 1992; Bischoff et al 1993). Furthermore Rantes has been shown to be expressed in human eosinophils, which are able to store and release the bioactive protein (Ying et al 1996), suggesting that this chemokine may function in an autocrine fashion. This C-C chemokine has also been shown to be expressed by a number of other cell types including fibroblasts, endothelial cells, astrocytes and renal tubular epithelium (Kiene et al 1993; Heeger et al 1992; Sticherling et al 1994; Noe et al 1996) as well as platelets (Kameyoshi et al 1992). Rantes is also expressed by activated epidermal and oral keratinocytes (Li et al 1996). Moreover dermal fibroblasts have recently been shown to release an amino-terminally truncated form of Rantes (Noso et al 1996).

In vivo the intradermal injection of Rantes has been shown to induce eosinophilic and monocytic intradermal infiltration, with histological evidence of eosinophilic activation (Meurer et al 1993). Rantes also promotes T cell trafficking to the site of chemokine injection (Murphy et al 1994) as well as promoting T cell homing to the thymus of SCID mice (Murphy et al 1994). These *in vivo* results suggest that Rantes may be involved in the pathogenesis of inflammatory disorders involving these cell types, and this is currently under intensive investigation by a number of groups. In fact Rantes has been implicated in diseases such as rheumatoid arthritis (Whyte and Binns 1994). Interestingly more recent studies suggest that Rantes, as well as MIP-1α and MIP-1β are endogenous HIV suppressive factors (Cocchi et al 1995). Furthermore since receptors that bind these C-C chemokines, including the

receptor CCR-5 have been shown to be involved in the entry of HIV into CD4+ cells, these chemokines may be able to prevent HIV entry into these cells by blocking the binding of the retrovirus to the receptor (Dragic et al 1996; Cairns and D'Souza 1998).

1.3.2.5. Myeloid Progenitor inhibitory Factors 1 and 2 (MPIF-1, MPIF-2).

Two novel human β-chemokines have been recently characterised as inhibitors of haemopoietic primitive cell proliferation (Patel et al 1997). The two polypeptides were identified in a cDNA sequencing project and isolated from aortic endothelium and activated monocyte libraries respectively. MPIF-1 is a 99 amino-acid polypeptide and is 51% homologous to MIP-1α. MPIF-1 appears to be a potent inhibitor of bone marrow low-proliferative potential colony forming cells, a committed progenitor that gives rise to granulocyte and monocyte lineages. MPIF-1 also appears to be chemotactic for T lymphocytes. MPIF-2 is a 93 amino-acid protein showing 42% identity to MIP-1α. It is also chemotactic for T lymphocytes and has been described as a potent inhibitor of high proliferative potential colony-forming haemopoietic stem cells (HPP-CFC). It is therefore now becoming clear that MIP-1α shares its haemopoietic proliferation inhibitory properties with other members of the chemokine family.

1.3.2.6. Other CC chemokines.

A number of other C-C chemokines have been described (Baggiolini et al 1997). These have not been as well characterised as the cytokines already described and include the chemokines TCA-3, MIP-1γ, HCC-1, and the genes C10 and fic. The TCA-3 (T-cell activation gene-3) gene was first identified by a subtractive hybridisation protocol as a gene that is transcriptionally upregulated following T-cell activation (Burd et al 1987). TCA-3 is secreted as a 14kDa glycoprotein (Wilson et al 1990). TCA-3 is a monocyte chemoattractant like most of the C-C chemokines and the TCA-3 gene is located at the C-C chemokine locus (Wilson et al 1990). Interestingly TCA-3 also appears to be chemotactic for neutrophils both in vitro and in vivo, a property which is more a feature of C-X-C chemokines (Luo et al 1994). TCA-3 has also been shown to induce a respiratory burst, granule release, the calcium flux and increased adhesiveness to extracellular matrix proteins (Devi et al 1995). Interestingly this chemokine also appears to have non-inflammatory activities since it has been shown to

induce adhesion to fibronectin, chemotaxis and proliferation in primary cultures of mouse renal mesangial cells (Luo and Dorf 1996).

MIP-1γ was recently cloned from a macrophage cell line Raw 264.7 (Poltorak et al 1995) as a cDNA that exhibits significant homology to other C-C chemokines. MIP-1γ has also been shown to be expressed by a murine Langerhans cell cell line XS 52 and by bone marrow dendritic cells (Mohamazadeh et al 1996a and b). Interestingly MIP-1γ, which has an expected size of 11.3kDa appears to be secreted by XS52 cells as a major 10.5kDa isoform and a minor 9kDa variant. This presumably reflects the potential heterogeneity of MIP-1γ proteins resulting from differential processing as has been observed with other chemokines. MIP-1γ appears to function through a G-protein coupled receptor and has been shown to induce a calcium flux. Furthermore it is chemotactic for T lymphocytes. Although this chemokine is not as yet well characterised it is interesting to note that this protein has been found to be constitutively expressed in a wide variety of tissues, and circulates in the blood of healthy mice at concentrations of approximately 90nM.

HCC-1, like MIP-1γ, has only been identified very recently and again although it is not as yet fully characterised it too is found in the peripheral blood at high concentrations (1-80nM) (Schulz-Knappe et al 1996). It was in fact identified from the haemofiltrate of patients undergoing dialysis for chronic renal failure and consists of 74 amino-acids with the characteristic four cysteine residues. It is structurally related to MIP-1α with which it is approximately 46% homologous. Unlike MIP-1α it has only weak activities on human monocytes in that it can induce an intracellular calcium flux and granule release at 100-1000nM. However it is not chemotactic for monocytes, and is totally inactive on T lymphocytes, neutrophils and eosinophils. Interestingly however HCC-1 has been shown to enhance the proliferation of myeloid progenitors with a similar efficacy to MIP-1α but a lower potency.

The gene C10 also encodes a C-C chemokine that was identified by the differential screening of GM-CSF treated and untreated bone marrow cells (Orlofsky et al 1991). This gene has also been mapped to the C-C chemokine locus, however it has a different genomic structure from the other C-C chemokines since this gene contains a novel second exon which encodes a highly charged 16 amino-acid sequence that is inserted into the amino-terminus of the molecule (Berger et al 1996). This cytokine is not yet well characterised.

In summary it is therefore clear that most if not all of the chemokines play an important role in inflammation and the regulation of the immune response. Many of these molecules appear to have similar or identical activites which implies a degree of functional redundancy. Furthermore like many other cytokines these molecules often have diverse activities. For example MIP-1α, which I will now discuss in greater detail, has been shown to not only be chemotactic for a variety of inflammatory cells, but to also regulate the proliferation of haemopoietic progenitors and clonogenic keratinocytes.

1.3.3.The Macrophage Inflammatory proteins: MIP-1α and MIP-1β.

The first C-C chemokine to be identified was the human macrophage inflammatory protein (MIP). This gene was initially called LD78 (Obaru et al 1986) and was cloned from human tonsillar lymphocytes. Shortly after this an 8kDa, acidic heparin-binding protein was purified from the conditioned media of a lipopolysaccharide stimulated murine macrophage cell line (Wolpe et al 1988). This protein was called Macrophage Inflammatory Protein-1 (MIP-1) since it was found to be a potent inflammatory mediator. Further biochemical analysis revealed that MIP-1 comprised two proteins, MIP-1α and MIP-1β. Partial amino-terminal sequencing confirmed that MIP-1α was the murine homolog of LD78.

1.3.3.1 The MIP-1α gene and its expression.

When the muMIP-1α cDNA was first cloned, after the purification of the murine protein, it was found to be 763 base pairs long comprising a short 5' non-coding region, an open reading frame of 276 nucleotides and a long 3' untranslated region which contains a polyadenylation signal and three repeats of an AT rich motif that may be involved in mRNA stability (Caput et al 1986; Davatelis et al 1988). Further investigation has shown that the MIP-1α and MIP-1β genes map to the C-C chemokine locus on human chromosome 17 in the region of q11 to q21 (mouse chromosome 11). Moreover MIP-1α and MIP-1β have highly homologous 5' flanking regions (over 2 kilobases upstream of the transcription start site). This suggests that the expression of these two proteins is co-regulated. In view of this only MIP-1α expression is discussed.

Human MIP-1α or LD78 has 75% sequence similarity to murine MIP-1α. There are at least two different LD78-like genes in the human genome (Nakao et al 1990). These have been termed LD78α and LD78β. They are both transcribed in a number of cell-lines (Nakao et al 1990). The coding regions of the LD78α and LD78β genes show up to 96% homology. However there are two amino-acid changes in the signal peptide sequence as well as three amino-acid substitutions in the mature protein. Two of the amino-acid changes in the mature protein are conservative serine to glycine changes. However there is a serine to proline change in position 2 at the amino-terminal end of the protein that may be of some functional significance.

LD78α

MQVSTAALAVLLCTMALCNQF*SASLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA

LD78β

MQVSTAALAVLLCTMALCNQVLSAPLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPSVIFLTKRGRQVCADPSEEWVQKYVSDLELSA

Interestingly Nakao et al (1990) suggested that according to Von Heijne's rule these amino-acid changes should both result in similar signal peptide cleavage sites. However Heijne's method for predicting signal sequence cleavage sites in fact indicates that while LD78β should be cleaved at the expected serine (position 23) to result in the amino-terminus APLAADTPT..., the LD78α gene will result in a different signal peptide cleavage site (the alanine at position 26), resulting in a -4 amino-terminally truncated protein with the amino-terminal sequence of ADTPTA... Interestingly Cocchi et al (1995) have recently purified a -4 amino-terminally truncated variant of LD78 from the supernatant of CD8+ T lymphocytes. They suggest that this is a true LD78 amino-terminus. This therefore indicates that human LD78α is probably secreted as a -4 amino-terminally truncated form, while human LD78β is secreted in a full-length form (GJ Graham: personal communication). Therefore while murine MIP-1α appears to only be secreted as a full-length 69 amino-acid protein, human MIP-1α (LD78) is probably secreted as both a 69 amino-acid protein (LD78β) and as a 65 amino-acid protein (LD78α).

These MIP-1α genes are single copy genes containing two introns and three exons (Grove et al 1990; Oppenheim et al 1991), a gene structure which is generally conserved throughout the chemokine family. Furthermore most haemopoietic mature cell types appear to be able to express MIP-1α. Macrophages (Davatelis et al 1988, Wolpe et al 1988), T cells as well as B cells (Yamamura et al 1989, Obaru et al 1986, Lipes et al 1988), mast cells (Burd et al 1989), eosinophils (Costa et al 1993), neutrophils (Kasama et al 1993) and epidermal Langerhans cells (Heufler et al 1992, Matsue et al 1992a, Xu et al 1995) have been shown to express MIP-1α mRNA. Non-haemopoietic cells have also been shown to express MIP-1α. Primary cultures of human fibroblasts (Nakao et al 1990), spermatogonia (Hakovirta et al 1994), astrocytes (Kim et al 1995) and a human glioma cell line U105MG (Nakao et al 1990) have been shown to express MIP-1α.

However in most of these cell types cellular stimulation is necessary to induce MIP-1α mRNA expression. Stimuli such as lipopolysaccharide (LPS), intercellular adhesion molecule-1

(ICAM-1), CD-40, TNF-α and Interferon-γ have all been shown to rapidly upregulate MIP-1α expression. On the other hand anti-inflammatory regulators such as TGF-β (Maltman et al 1993), IL-4 (Standiford et al 1993) and IL-10 (Kasama et al 1994) have been shown to downregulate the production of MIP-1α transcripts. MIP-1α expression appears to be closely controlled and although these regulatory mechanisms are not fully understood they are probably complex.

1.3.3.2 The MIP-1 Proteins: Structure and Function.

The products of the MIP-1 genes are both secreted heparin-binding proteins. These genes encode primary translation products of 92 amino acids, including typical amino-terminal signal sequences. In both murine MIP-1α and MIP-1β the signal peptide is cleaved after a serine residue to generate mature proteins 68-69 amino-acids long. The mature secreted forms of both murine and human MIP-1α and MIP-1β are approximately 70% homologous and run as 8kDa bands on an SDS-PAGE gel. The proteins have the characteristic four cysteines that form two disulphide bonds that are critical to the protein's structure and function. Disulphide bridges link the first cysteine to the third and the second cysteine to the fourth (Reviewed by Schall 1991).

The CC and CXC chemokines have similar secondary structures (Schall 1991). In fact structural analysis of both MIP-1α and MIP-1β has shown that these proteins have similar secondary and tertiary structure to the CXC chemokines IL-8 and PF-4 (Lodi et al 1994; Clements et al 1992) with which they share limited sequence homology. The three dimensional structure of MIP-1β has been resolved by multidimensional nuclear magnetic resonance (Lodi et al 1994). Since this protein, like a number of other members of the chemokine family has a tendency to form large multimeric aggregates (discussed below) at higher concentrations, the studies were performed at a low pH where MIP-1β is a dimer. These studies have shown that with the exception of the amino-terminus which appears to be flexible and partially disordered, the MIP-1β structure is well defined. The main secondary structure is a triple- stranded antiparallel β-sheet arranged in a greek key conformation on which lies an α-helix. The amino-terminus comprises an irregular strand and a series of turns that form a long loop extending from residues 12-20. This is followed by a four residue helical turn which leads into strand β1. This structure is very similar to that defined for

another C-C chemokine Rantes (Skelton et al 1995). Furthermore at the monomer level there are many similarities in the tertiary structures of MIP-1β and the CXC chemokine IL-8 (Clore et al 1990; Baldwin et al 1991). There are however a number of differences that may result in the different receptor specificities of the two major chemokine families. These include differences in the direction of the amino-terminal residues preceding the first cysteine as well as differences in the conformation of the first disulphide bridge which is caused by the extra amino-acid between the two cysteines in IL-8. Furthermore the α-helix in IL-8 extends 5 amino-acids longer than in MIP-1β at the carboxy terminus and the turn between strands β1 and β2 also differ in conformation at residues 46 and 47.

Although the tertiary structures of MIP-1β and IL-8 are similar, the quaternary structures that result from protein aggregation are quite different. Since CXC chemokines do not generally bind CC chemokine receptors, and vice-versa CC chemokines do not bind CXC receptors (despite considerable promiscuity in receptor binding in the individual families), these major differences in the dimeric CXC and CC chemokine structures have been postulated to explain the distinct receptor binding profile of these two subfamilies (Lodi et al 1994). Furthermore it has been suggested that the large concave surface of the MIP-1β dimer is involved in the receptor binding of the C-C chemokines. This hypothesis may not however be correct since there is now evidence to suggest that these chemokines function as monomers.

1.3.3.3. Aggregation.

Most, if not all, of the chemokines identified so far have a tendency for spontaneous aggregation to form homodimers and larger multimeric aggregates. In fact platelet factor 4 and interleukin 8, whose crystal structures are well characterised (St Charles et al 1989; Baldwin et al 1991) have been shown to exist as dimers, with platelet factor 4 also forming tetrameric aggregates. Interestingly some of these molecules including MIP-1α and MIP-1β can form large multimeric aggregates of very high and variable molecular weights at higher concentrations *in vitro*. The physiological relevance of this aggregation is uncertain, and although it has been suggested that this phenomenon may help concentrate the chemokine at its site of action it is probably more likely that this multimerisation is an artefact related to the high concentrations of the protein used *in vitro*. To investigate this non-aggregating chemokine molecules have been generated by different investigators. A chemically

synthesised non-aggregating monomeric form of IL-8, N-methyl-Leu-25 IL-8 has been shown to be fully functional in a number of different assays (Rajarathnam et al 1994). Furthermore a number of non-aggregating mutants of MIP-1α have also been generated (Graham et al 1994). These mutants termed PM1, PM2 and PM3 were generated by systematically and conservatively neutralising carboxy-terminal acidic amino-acid residues using PCR based site-directed mutagenesis. These mutants PM1, PM2 and PM3 were found to display molecular weights corresponding to the tetramer, dimer and monomer of MIP-1α respectively. Interestingly all three mutants are equipotent with the wildtype molecule in both the haemopoietic CFU-A assay and monocyte shape change assays. These mutagenesis studies suggest that aggregation is not necessary for activity and indicate that aggregation is a dynamic and reversible phenomenon resulting from electrostatic interaction (Graham et al 1994). Conversely however there is some evidence to suggest that the C-C chemokine MCP-1 interacts with its receptor as a homodimer (Zhang and Rollins 1995).

1.3.3.4 Structure-Function Studies.

The above studies suggest that MIP-1α is active in a monomeric form. Therefore the differences in quaternary structure between the CXC and CC chemokines are unlikely to explain the disparate receptor binding profiles of these two subfamilies. Since the tertiary and secondary structures of these molecules are similar, it is therefore likely that receptor selectivity is due to the primary amino-acid sequence of the peptides, with the amino-terminus being particularly important (Reviewed by Wells et al 1996; Baggiolini et al 1997). It is interesting to note therefore that an analysis of the hydrophobic residues in the CC and CXC chemokines has shown that although the core hydrophobic residues of the two families are at equivalent positions, which is in keeping with their similar tertiary structures, the surface hydrophobic clusters are located in very different regions in the two sub-families. This is not only in keeping with the observed differences in quaternary structure, but may help partially explain the general lack of receptor cross-binding and reactivity between the two sub-families (Covell et al 1994).

To further delineate the residues which define these differences, a number of other studies have utilised sequence comparisons of the CXC and CC families, as well as the observed three dimensional structures of the chemokines, to guide site-directed mutagenesis studies.

The mutants generated by these studies have helped to delineate the amino-acids in the chemokine molecule which contribute to the molecule's activity.

CXC FAMILY

IL-8 SAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEI IVKLSD. GRELCLDPKENWVQRVVEKFLKRAENS

NAP-2 AELRCMCIKTTSG ..IHPKNIQSLEVIGKGTHCNQVEVIATLKD. GRKICLDPDAPRIKKIVQKKLAGDESAD

GROα ASVATELRCQCLQTLQG.IHPKNIQSVNVKSPGPHCAQTEVIATLKN. GRKACLNPASPIVKKIIEKMLNSDKSN

IP-10 VPLSRTVRCTCISISNQPVNPRSLEKLEIIPASQF CPRV EI IATMKKKGEKRCLNPESKAIKNLLKAVSKEMSKRSP

PF-4 EAEEDGDLQCLCVKTTSQ. VRPRHITSLEVIKAGPHCPTAQLIATLKN. GRKI CLDLQAPLYKKIIKKLLES

C-C FAMILY

MIP-1α ASLAADTPTAC.CFSYTSRQI PQNFIA DYF ETSSQ CSKPGV IFLTKRS. RQV CADPSEEWVQKYVSDLELSA*

MIP-1β APMGSDPPTACCFSYTARKLPRNFVVDYYETSSL CSQPAVVFQTKRS.KQV CADPSESWVQEYVYDLELN *

RANTES SPYSSDT. TPCC FAYIARPLPRAHIK E YFYTSGK CSNPAVVFVTRKN.RQVCANPEKKWVREYINSLEMS

MCP-1 QPDAINAPVT CC YNFTNRKISVQRLAS YRRITSSK. CPKEAVI FKTIVA. KEI CADPKQKWVQDSMDHLDKQTQTPKT

**** Human proteins***

IL-8 was one of the first chemokines to be analysed in structure-function studies. Initial work focused on determining the importance of the amino- and carboxy- termini in neutrophil chemotaxis and exocytosis (Clark-Lewis et al 1991). Analogs of IL-8 were chemically synthesised and their activity compared with that of the full-length molecule. These investigations showed that the amino-terminal residues 4, 5 and 6 of IL-8 are critically important for IL-8 receptor binding and therefore chemokine activity. Moreover, studies with amino-terminally truncated forms of IL-8 show that IL-8 analogs with deletions or mutations of the ELR motif can function as antagonists of the wildtype molecule (Moser et al 1993).

IL-8 (1-72)	SAL**ELR**CGC.....	Wildtype
IL-8 (4-72)	**ELR**CGC.....	2-5 fold higher potency than 1-72.
IL-8 (5-72)	**LR**CGC.....	Binds receptor weakly, partial agonist.
IL-8 (6-72)	**R**CGC.....	Binds receptor weakly. Receptor antagonist.
IL-8 (7-72)	CGC.....	Does not bind IL-8 receptor.
IL-8 AAR(7-72)	AA**R**CGC.....	Binds receptor. Antagonist.

It is therefore clear that for IL-8 the ELR motif in the amino-terminus is crucial for the biological activities of this CXC chemokine. This tripeptide ELR motif (underlined in the

sequences above) is not however the only determinant of IL-8 activity since an amino-terminal decapeptide lacks both IL-8 activity and receptor binding. On the other hand although synthetic analogs shortened at the carboxy-terminus are generally less potent than the full-length molecule, a mutant (1-51) lacking the terminal 21 amino-acids, is still active suggesting that the carboxyl terminus of IL-8 is not crucial for receptor binding. Interestingly however, while the 1-51 analog is approximately 50-fold less potent than the wild-type molecule, addition to this mutant of a peptide corresponding to the carboxy-terminal 22 amino-acids results in a two-fold increase in 1-51 activity suggesting that this portion of IL-8 may be important in stabilising the three-dimensional structure of IL-8. These studies also showed that one of the carboxy-terminal mutants, which was shorter than the wild-type by three amino-acids, 1-69 was in fact two-fold more potent than the full-length molecule, although further truncation by another three amino-acids, which generates the mutant 1-66, results in an analog that is equipotent to IL-8. Independent studies with the CXC chemokine NAP-2, which is generated by proteolytic processing from PBP and β-thromboglobulin, have also shown that limited carboxy-terminal truncation of NAP-2 can generate variants with modestly enhanced activity (Brandt et al 1993; Ehlert et al 1995). What is however particularly interesting about these studies is that a carboxy-terminal truncated form of NAP-2 was identified in the culture medium of activated peripheral blood mononuclear cells (PBMC). This variant NAP-2(1-66) which is four amino-acids shorter than the full-length molecule is 3-4 fold more potent than NAP-2, leading the investigators to suggest that limited truncation at the carboxy-terminus may be a physiological mechanism for fine-tuning chemokine activity. It is interesting to note here that further truncation beyond the 1-66 resulted in decreased NAP-2 activity. These investigations overall indicate that the carboxy-terminus of these molecules is not essential for their activities, although limited carboxy-terminal truncation may result in in a modest but significant increase in chemokine activity. Furthermore the amino-terminus of these molecules appears to be critical, although not sufficient, for biological activity.

Overall these results indicate that there must therefore be additional critical binding sites in the IL-8 molecule between the cysteine at position 8 and the cysteine at position 50. Experiments utilising rabbit IL-8, which shares 82% sequence identity with human IL-8, but has a 200-fold lower affinity for the IL-8A receptor, shed further light on this. It was found that

simply replacing two amino acids (histidine 13 and threonine 15) in rabbit IL-8 with the corresponding amino-acids of the human protein (tyrosine 13 and lysine 15) converts the low-affinity binding of rabbit IL-8 to the high affinity binding of the human protein. Therefore this region of the IL-8 molecule also appears to be critically important in IL-8 receptor binding, either through directly interacting with the IL-8A receptor or indirectly by determining the conformation of this chemokine (Schraufstatter et al 1995).

A different approach designed to study the importance of other residues in the IL-8 and IL-8 receptor interaction involves incorporating the ELR motif into chemokines that do not possess IL-8 activity (Clark-Lewis et al 1994). It is clear from a number of studies that some of the members of the CXC chemokine sub-family lack the activity of IL-8 on neutrophils. Moreover these molecules, which include IP-10, mig and PF-4 (see table above), lack the ELR motif and have quite different activity profiles to IL-8. A series of experiments have shown that the insertion of the ELR tripeptide motif into PF-4 converts this variant of PF-4 into a molecule with IL-8 like activity. However insertion of the ELR motif is not sufficient to introduce IL-8 activity into IP-10, which indicates again that the ELR is necessary but not sufficient for IL-8 receptor binding. Further studies involving a series of mutants in which hybrids were derived by substituting regions of IL-8 into IP-10, have shown that other regions of the IL-8 molecule are important for its biological activities. These include the residues at positions 30-35, particularly the glycine-proline motif, which defines an atypical β-turn, as well as the region 10-22, which although less critically important includes a number of residues that were seen to be important in IL-8 receptor binding (Tyrosine 13, Phenylalanines at positions 17 and 21). It is likely that the glycine-proline motif is important to provide a structural scaffold for the receptor binding amino-terminal region which includes the ELR motif and secondary binding and conformational determinants between residues 10 to 22 (Clark-Lewis et al 1994).

The amino-terminus of the C-C chemokines is also critical to the receptor binding of these molecules. The C-C chemokine Rantes is a potent chemotactic and activating agent for a number of leukocytes including T-cells. When overlapping 10 amino-acid peptides scanning the whole length of the protein were synthesised and bioassayed, it was found that the Rantes decapeptides (1-10), (3-12) and (5-14) were all chemotactic for T-cells suggesting that this region of the molecule is sufficient for this Rantes T-lymphocyte chemotaxis.

Peptides from other regions of the Rantes molecule were inactive (Wells et al 1995). None of the peptides were able to give a calcium flux in THP-1 cells, unlike the parent molecule, suggesting that the amino-terminal region of Rantes is not sufficient for this activity. Independent results have also shown that extension of the Rantes molecule by the retention of an amino-terminal initiating methionine produces a Rantes analog, Met-Rantes that is not only inactive but is also a potent antagonist of the wildtype molecule in calcium mobilisation studies and chemotaxis assays performed on the promonocytic cell line THP-1 (Proudfoot et al 1996). This indicates that the integrity of the amino-terminus of Rantes is critical to its receptor binding.

Separate studies on another C-C chemokine MCP-1, have also shown that the amino-terminus of MCP-1 is crucial for this protein's activities. A number of chemically synthesised amino-terminally truncated mutants of MCP-1 were generated and bioassayed on peripheral blood monocytes and THP-1 cells. The results obtained (see overleaf) were somewhat surprising but again confirm that the amino-terminus of MCP-1 is a critical determinant of MCP-1 receptor binding and activity (Gong and Clark-Lewis 1995). Although these observations have parallels with IL-8, the situation with MCP-1 is obviously more complex. Firstly, while the nature of the amino-terminal residue is critical, the nature of the amino-terminal sidechain can be varied since asparagine and other non-polar residues of various types can substitute for the pyroglutamate found in the wildtype molecule. Moreover other studies have shown that the deletion of the amino-terminal residue of MCP-1 dramatically changes the activities observed for this molecule. Therefore while the wildtype molecule is a potent basophil activator with no effect on eosinophils, the 2-76 molecule is a potent eosinophil chemoattractant with little effect on basophils (Weber et al 1996). These results indicate that the amino-terminus amino-acid is critical in determining the function of this molecule. Furthermore deletion of the first two amino-acids results in total loss of activity, which is in keeping with this. However further deletion results in the regaining of activity, with a molecule that is only fourfold less active than the full-length chemokine in monocyte studies.

MCP-1	*QPDAINAPVTCC.....	Wildtype activity (* ≡ pyroglutamate).
MCP-1 (Ac)	AcQPDAINAPVTCC.....	300fold less active(Ac ≡ Acetylation).
MCP-1(Ala^{-1})	AQPDAINAPVTCC.....	300fold less active.
MCP-1(Asn^{1})	NPDAINAPVTCC.....	Fully active.
MCP-1($AcAsn^{1}$)	AcNPDAINAPVTCC.....	Considerably reduced activity.
MCP-1(Asp^{1})	DPDAINAPVTCC.....	Inactive.
MCP-1(Ala-1)	APDAINAPVTCC.....	Fully active.
MCP-1(2-76)	PDAINAPVTCC.....	300 fold less potent than MCP-1.
MCP-1(3-76)	DAINAPVTCC.....	Inactive.
MCP-1(4-76)	AINAPVTCC.....	Inactive.
MCP-1(5-76)	INAPVTCC.....	Active but 4fold less potent than MCP-1.
MCP-1(6-76)	NAPVTCC.....	Virtually inactive but still binds receptor.
MCP-1(7-76)	APVTCC.....	Inactive. Binds and desensitises receptor.
MCP-1(8-76)	PVTCC.....	Inactive. Antagonist of MCP-1(IC_{50}=60nM)
MCP-1(9-76)	VTCC.....	Inactive. Antagonist of MCP-1(IC_{50}=20nM)
MCP-1(10-76)	TCC.....	Inactive. Weak antagonist (IC_{50}=600nM)
MCP-1(11-76)	CC.....	Inactive. Weak antagonist (IC_{50}=1μM)

These studies suggest that the amino-terminal residues are not essential for signalling, but may be necessary for optimal binding Unlike the ELR in IL-8, the functionally critical amino-terminal region of MCP-1 appears to extend over all of the first 10 amino acids of this CC chemokine. These studies also suggest that like IL-8, MCP-1 must have other receptor binding sites outside the amino-terminus. Independent studies have in fact shown that the tyrosine residue at position 28 is very important in receptor binding of MCP-1 (Zhang et al 1994). Interestingly this residue is conserved throughout the C-C family, and the corresponding residue in the CXC (Leucine 25) is also highly conserved suggesting that the residue at this position may help define the receptor specificities of the two sub-families. This has been analysed by mutating leucine in the CXC chemokine IL-8 to the tyrosine typical of the CC family. This mutation imparts to IL-8 a novel monocyte chemoattractant activity and decreases the molecule's potency in neutrophil chemoattractant assays, confirming the importance of this region in not only IL-8 receptor binding but also in conferring specificity

between CXC and CC chemokines (Lusti-Narasimhan et al 1995 and 1996; Reviewed by Wells et al 1996). Other investigations indicate that a number of other sites may be involved in C-C chemokine receptor binding. The arginine at position 24 in MCP-1 has been seen to play a part in MCP-1 activity since its mutation to phenylalanine results in a molecule with only 5% of wildtype activity, while deletions of the carboxy terminal α-helix resulted in a molecule that possessed ~10% of MCP-1 function suggesting that as for IL-8, the carboxy-terminus of MCP-1 is not essential, but may be required for optimal activity (Zhang et al 1994).

Finally recent studies on MIP-1α have shown that a non-heparin binding mutant of MIP-1α called HepMut, which was generated by the neutralisation of two basic amino-acids in the region between the two carboxy-terminal cysteines, does not bind the MIP-1α receptor CCR1 (Graham et al 1996). This does not appear to be related to the loss of heparin-binding activity since the wild-type molecule is still able to bind CCR1 in glycosaminoglycan deficient CHO cell lines. This suggests that this region of the CC chemokine MIP-1α is also critical to CCR-1 receptor binding.

1.3.3.5 MIP-1α and its Mode of Action: Receptors.

The means by which MIP-1α exerts its manifold activities on its different target cells are still as yet poorly characterised. However several receptors that bind this and other chemokines have been cloned by molecular techniques and studies on these receptors are now beginning to shed light on the mechanisms of action of these molecules (Reviewed by Baggiolini et al 1997). These receptors have seven-transmembrane domains and form a distinct group of structurally related proteins within the large family of G-protein coupled receptors. They have conserved transmembrane region structural motifs as well as two conserved cysteine residues, one in the amino-terminal domain and the other in the third extracellular loop. These cysteines form a disulphide bond that is crucial for the conformation of the ligand-binding site.

The CXC and CC chemokine receptors form two different subgroups with significant sequence conservation (CXC receptors: 36-77%; CC receptors: 46-89%), specific chromosomal localisation (CXCR genes located on chromosome 2q; CCR genes located on chromosome 3p) and sub-group specific binding profiles. The first chemokine receptors to be identified were the neutrophil receptors for the CXC chemokine IL-8, IL-8RA and IL-8RB (Holmes et al 1991; Murphy et al 1991), now known as CXCR1 and CXCR2 respectively. The first C-C chemokine receptor to be cloned was CCR1 (Neote et al 1993), which was isolated from HL60 cells treated with PMA. This receptor has been shown to bind MIP-1α and Rantes, as well as MCP-2, MCP-3 and MCP-4 (Uguccioni et al 1996; Franci et al 1995; Ben-Baruch et al 1995; Combadiere et al 1995). A receptor for MCP-1 designated CCR2 occurs in two RNA-spliced variants CCR2a and CCR2b with different carboxy-terminal regions which alter signal transduction but not ligand binding (Charo et al 1994; Myers et al 1995). CCR3, the eotaxin receptor, is expressed primarily by eosinophils and binds Eotaxin as well as Rantes and MCP-3 but not MIP-1α or MIP-1β (Daugherty et al 1996). CCR4 binds the recently described chemokines TARC and MDC (Imai et al 1997, 1998). CCR5 also binds MIP-1α, as well as MIP-1β and RANTES (Samson et al 1996). Studies on the HIV inhibitory activities of MIP-1α, RANTES and MIP-1β have shown that CCR5 functions as a required co-receptor for the entry of M-tropic HIV-1 into CD4+ cells (Dragic et al 1996). Further work has shown that a number of other chemokine receptors serve as co-receptors that are necessary for the entry of HIV and related lentiviruses into the cell (Cairns and D'Souza 1998). These include

CCR2 (Doranz et al 1996; Choe et al 1996), CCR3 (Doranz et al 1996; Choe et al 1996), CCR8 (Tiffany et al 1997; Horuk et al 1998) as well as CXCR4 (Feng et al 1996) and the fractalkine receptor CX3CR (Reeves et al 1997). The discovery of this relationship between chemokine receptors and HIV has generated considerable interest in the potential use of chemokines or chemokine variants in the treatment and prevention of HIV infection (Cairns and D'Souza 1998). A number of other chemokine receptors have been identified including the promiscuous CC chemokine receptor D6 which, although specific for the β-chemokine family, binds the majority of CC chemokines (Nibbs et al 1997 a and b).

Most of the chemokine receptors identified so far are G-protein coupled receptors.They can be sub-classified into four general main categories (Schall and Bacon 1994): specific receptors, shared receptors, promiscuous receptors and virally encoded receptors. Specific receptors bind only one chemokine and are specific to that molecule. IL-8RA is thought to be specific for IL-8 (Lee et al 1992). Shared receptors, which include CCR-1, are able to bind more than one chemokine. Most of the chemokine receptors identified so far are shared receptors. CCR-1 can in fact bind MIP-1α, Rantes, MIP-1β and MCP-1. The Duffy blood group erythrocyte antigen has been shown to be a promiscuous chemokine receptor because it is able to bind both CXC and CC chemokines (Neote et al 1994). It has also been shown to be involved in the binding of the malaria pathogen *Plasmodium vivax* to red blood cells (Horuk et al 1993). Interestingly the Duffy receptor does not seem to be able to signal through G protein-coupled mechanisms (Neote et al 1994) and its significance remains uncertain. Since there are a large number of Duffy receptors on an erythrocyte, it has been suggested that the Duffy antigen may function as a 'sink' and mop up excess chemokine from the peripheral circulation. Finally the fourth class of chemokine receptors is known as the virally encoded chemokine receptors. These include a gene product from cytomegalovirus known as US28 which encodes a functional C-C chemokine receptor. US28 is approximately 30% homologous to the known chemokine receptors and is able to bind MIP-1α, Rantes, and MCP-1 as well as transduce a signal via a calcium mobilisation pathway (Gao et al 1994). A gene product from Herpesvirus samiri has also been shown to bind CXC chemokines and share over 30% homology with the IL-8 receptors (Ahuja et al 1993). The biological role of these viral proteins is not known, but it is likely that these receptors are involved in the pathogenesis of infection with these viral pathogens.

1.3.3.6. MIP-1α and its Mode of Action: Signal Transduction.

The signalling mechanisms by which MIP-1α exerts its activities are not well understood. However it is clear that the effects of MIP-1α on a number of cell types can be abrogated by the pretreatment of the cells with *Bordatella pertussis* toxin, which inhibits signal transduction through a G protein termed $G\alpha_i$ (McColl et al 1993). This indicates that at least a proportion of MIP-1α's activities are mediated through G-protein coupled receptors. MIP-1α has also been shown to induce a calcium flux in a number of target cells. This rapid and transient rise in intracellular calcium levels is sensitive to inhibition by pertussis toxin and is probably a result of the increased inositol triphosphate release by the G-protein activated enzyme phospholipase C. A considerable variety of downstream cascades have been reported to be activated by these operative G-proteins. These include phospholipase C (PLC), adenyl cyclase which generates cAMP, as well as phospholipase A_2. MIP-1α has also been shown to activate phospholipase A_2 in monocytes (Locati et al 1994), which in turn induces a rapid release of arachidonic acid that is thought to be important in chemotaxis. Moreover investigations on the myeloid leukaemia derived cell line MO7e indicates that MIP-1α treatment does indeed result in an increase in phosphatidylcholine levels and cAMP implicating phospholipase C and adenyl cyclase in the inhibition of MO7e cell proliferation (Mantel et al 1995). Phospholipase C generates diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG can then activate protein kinase C, while IP_3 releases the intracellular calcium stores, which results in the calcium flux. Moreover since the generation of cAMP by adenyl cyclase may be an inhibitory stimulus to haemopoietic cell proliferation, it has been suggested that this is the mechanism by which MIP-1α regulates haemopoietic progenitor proliferation (Mantel et al 1995). The mechanisms by which cAMP functions to generate these inhibitory activities remain unclear. However cAMP has been shown to inhibit the interaction of the two proto-oncogene products ras and raf (Cook et al 1993), which results in the uncoupling of one of the major mitogenic signalling pathways, the mitogen activated kinase (MAPK) pathway (Wu et al 1993). Other studies suggest that MIP-1α, by increasing intracellular cAMP levels, can block the activation of Raf-1 kinase, a component of the MAPK signalling pathway, induced by growth factors (Aronica et al 1995). Since growth factors such as GM-CSF (Miyazawa et al 1991) and EGF (Wu et al 1993) have been shown

to function through this pathway, this suggests that MIP-1α may inhibit cellular proliferation induced by such cytokines through this mechanism.

1.3.3.7 Biological Activities of MIP-1α: MIP-1α and the Immune Response.

MIP-1α has been characterised primarily as an inflammatory cytokine. It is a potent chemoattractant and activator for a number of different inflammatory cell types *in vitro*. MIP-1α is a potent monocyte chemoattractant (Schall et al 1990) as well as a modulator of macrophage function (Fahey et al 1992). MIP-1α has also been shown to chemoattract distinct populations of lymphocytes (Taub et al 1993; Schall et al 1993). These studies have shown that MIP-1α can attract both CD4+ and CD8+ T lymphocytes, and B cells as well as regulate lymphocyte activation (Taub et al 1996). MIP-1α has also been shown to be able to induce the migration and activation of eosinopils (Rot et al 1992), and of basophils and mast cells (Alam et al 1992; Bischoff et al 1993). The activities displayed by this chemokine *in vitro* suggest that MIP-1α may be involved in the pathogenesis of a number of inflammatory and allergic disorders (Baggiolini and Dahinden 1994). MIP-1α has in fact been implicated in the pathogenesis of a number of inflammatory lung diseases including pulmonary fibrosis (Standiford et al 1993). MIP-1α has also been shown to be involved in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), which is a murine model for the demyelinating disease multiple sclerosis (Karpus et al 1995). These studies have shown that specific antibody to MIP-1α can significantly ameliorate the severity of both EAE and bleomycin induced lung injury suggesting that this molecule may be involved in the autoimmune process that results in the tissue injury of these disease processes. Interestingly data obtained from MIP-1α knockout mice indicates that MIP-1α is necessary for the development of the autoimmune myocarditis induced by coxsackie viral infection (Cook et al 1995). These results together indicate that MIP-1α is a major player in the pathogenesis of inflammatory processes in vivo, with perhaps a specific role in the initiation of autoimmune reactions. Moreover MIP-1α knockout mice have also been shown to have an altered response to Influenza viral infection resulting in a delayed clearance of this pathogen, suggesting that this chemokine may be involoved in the immune response to this viral infection. On the other hand however MIP-1α knockout mice have apparently normal

haemopoiesis. No obvious differences in peripheral blood or bone marrow differentiated and primitive blood cells are observed in these mice, with apparently normal spleen and lymph node lymphocyte numbers.

1.3.3.8 Biological Activities of MIP-1α: The Regulation of Cell Proliferation.

a) Haemopoiesis.

MIP-1α is now well characterised as an inhibitor of primitive haemopoietic cell proliferation. Prior to the identification of this chemokine, a number of studies had shown that an extract of bone marrow contains an inhibitor of both *in vitro* and *in vivo* haemopoietic stem cell assays (Lord et al 1976). Furthermore studies showed that the bone marrow macrophage is a likely source of this inhibitor, and that the macrophage cell line J774.2 produces a similar inhibitory activity. This activity was purified and when characterised by amino-terminal sequencing and DNA cloning, was found to be due to the chemotactic cytokine MIP-1α (Graham et al 1990). MIP-1α is able to inhibit growth factor mediated colony formation by CFU-A haemopoietic progenitors at picomolar to low nanomolar concentrations. It also inhibits haemopoietic progenitors from undergoing DNA synthesis. Furthermore MIP-1α also inhibits day 12 CFU-S haemopoietic progenitors *in vivo*. MIP-1α does not however have any inhibitory effects on the proliferation of the more committed GM-CFC progenitors (Graham et al 1990).

This inhibitory activity has been confirmed by a number of other groups and studies have shown that recombinant murine MIP-1α can inhibit the *in vitro* colony formation of a number of different primitive haemopoietic progenitors, including CFU-GM, BFU-E and CFU-GEMM (Broxmeyer et al 1990; Eaves et al 1991). Furthermore the increase in CFU-S induced by *in vitro* culture in the presence of IL-6 or IL-3 is reduced in the presence of MIP-1α. The growth-factor stimulated proliferation of the multipotential haemopoietic FDCP-mix A4 cell line has also been shown to be inhibited by MIP-1α (Lord et al 1992). Furthermore in long-term bone marrow cultures MIP-1α has been shown to be able to inhibit the ability of fresh medium to stimulate entry into S-phase of primitive high proliferative potential clonogenic cells in this system (Eaves et al 1993). On the other hand more differentiated progenitors with low proliferative potential are not inhibited and there is in fact conversely some evidence to suggest that the proliferation of more mature progenitors is enhanced by MIP-1α (Broxmeyer

et al 1990). This bimodal form of activity has also been observed with a number of other negative regulators including TGF-β (see above).

Overall these studies show that MIP-1α can inhibit the proliferation of a variety of primitive haemopoietic cell types. Other studies however suggest that MIP-1α, unlike TGF-β, is not an inhibitor of the most primitive stem cell, although it does undoubtedly inhibit the proliferation of early haemopoietic progenitors. These investigations which include complex competitive repopulation studies indicate that MIP-1α does not inhibit the haemopoietic long-term repopulating cell (Quiesnaux et al 1993; Soma et al 1996). Moreover MIP-1α, unlike TGF-β, does not inhibit proliferation in the HPP-CFC-1 assay, which is thought to detect primitive stem cells. Furthermore separate studies have also shown that in stroma non-contact long-term bone marrow cultures, MIP-1α does not inhibit the proliferation of the most primitive cells in this system, the long-term bone marrow culture initiating cells (LTBMC-IC). Surprisingly however MIP-1α has been shown to be able to prolong the maintenance of the LTBMC-IC in long-term bone marrow cultures (Verfaillie et al 1994). The mechanism of this activity may be the result of a direct interaction of MIP-1α with the LTBMC-IC, but appears to be dependent on an uncharacterised effect of the stroma on the function of MIP-1α. It has been suggested that the stromal cell may activate the MIP-1α by producing an accessory factor. However structural activation of the MIP-1α molecule by the stromal cell has not been excluded. The effects of MIP-1α on the most primitive haemopoietic cell population is therefore not as yet fully defined.

However it is clear that MIP-1α does inhibit the proliferation of early haemopoietic progenitors including the CFU-S (Cooper et al 1994). Furthermore MIP-1α can also protect this population of cells from the cytotoxicity of anti-cancer chemotherapy *in vivo* (Dunlop et al 1992; Lord et al 1992; Lord et al 1995). Dunlop et al (1992) have demonstrated protection of the CFU-S/CFU-A compartment from the double injections of the cell-cycle-specific drug cytosine arabinoside, while Clements et al (1992) have shown that the *in vivo* injection of MIP-1α into mice protects the CFU-S from subsequent killing by tritiated thymidine. Lord et al (1992) have also demonstrated that MIP-1α can protect the CFU-S from the toxicity of another cell-cycle specific drug hydroxyurea. Further studies have shown that MIP-1α also

speeds up the recovery of thse primitive haemopoietic progenitors after cytotoxic therapy (Lord et al 1995).

However most anti-cancer chemotherapy regimes usually involve combinations of a number of different cytotoxics with different mechanisms of action. These drug combinations usually include non-cycle specific drugs. There is however *in vivo* evidence to suggest that MIP-1α can ameliorate the myelotoxicity of the non-cycle specific drug cyclophosphamide in mice (Parker et al 1995). If this is confirmed then MIP-1α may have a potential role in the amelioration of the myelotoxicity induced by cytotoxic chemotherapy since this is the main dose limiting factor in most cytotoxic regimens. MIP-1α has also been shown to be unable to inhibit the proliferation of chronic myeloid leukaemic progenitors (Eaves et al 1993), therefore this molecule may also be potentially useful in the purging of tumour cells ex vivo from stem cell harvests.

If MIP-1α is to be a useful therapeutic agent it must be non-toxic. MIP-1α is a potent inflammatory mediator and this activity has the potential to cause considerable side-effect. The use of the non-heparin binding mutant HepMut may however resolve this concern since this mutant has been shown to retain the stem cell inhibitory functions of MIP-1α, while apparently having no activity on monocytes *in vitro* (Graham et al 1996). Moreover if MIP-1α is to be useful as a myeloprotective agent, it must not at the same time inhibit the proliferation of neoplastic cells since this would hinder the treatment of the malignancy. The activity of MIP-1α on a wide variety of cell lines has therefore been evaluated and the results indicate that MIP-1α does not have any significant inhibitory activity on these clonogenic cancer cells (Korfel et al 1994; Parkinson E.K.- personal communication).

A MIP-1α variant BB10010 has been investigated in clinical phase I and II trials. These trials indicate that although MIP-1α is safe, and is able inhibit primitive haemopoietic progenitors in cancer patients (Broxmeyer et al 1998), it is however unable to reduce the toxicity of cytotoxic chemotherapy (Bernstein et al 1997).

b) MIP-1α and Spermatogenesis.

MIP-1α has also been shown to inhibit the proliferation of a number of non-haemopoietic clonogenic cell types. MIP-1α is constitutively expressed in the seminiferous epithelium and

has been recognised to be an inhibitor of the proliferation of DNA synthesis in primitive spermatogonia *in vitro* (Hakovirta et al 1994). Paradoxically MIP-1α increases the DNA synthesis of more mature and differentiated spermatogonia. These observations suggest that MIP-1α may be a physiological regulator of spermatogenesis. It is however interesting to note that MIP-1α knockout mice appear to have normal fertility. This suggests that MIP-1α is not essential for normal spermatogenesis.

c) MIP-1α and Clonogenic Epidermal Keratinocyte Proliferation.

MIP-1α has also been shown to be expressed in the epidermis. Studies investigating epidermal cytokine mRNA expression by reverse-transcriptase polymerase chain reaction (RT-PCR) technology, has revealed that MIP-1α is expressed in the epidermis and that the only detectable source of this cytokine in the epidermis is the Langerhans cell (Heufler et al 1992; Matsue et al 1992a,b and c). This is perhaps not surprising since MIP-1α has been shown to be expressed by a wide variety of haemopoietic cell types, and epidermal Langerhans cells are immunologically immature dendritic cells derived from the haemopoietic stem cell (Frelinger et al 1979). However while most haemopoietic cell types require some form of stimulation to express this cytokine, in the epidermis MIP-1α is expressed constitutively by the Langerhans cell, as seen by both RT-PCR and Northern blotting (Heufler et al 1992; Matsue et al 1992; Parkinson et al 1993). Keratinocytes on the other hand do not express MIP-1α constitutively. Furthermore inflammatory stimuli do not appear to induce MIP-1α expression in keratinocytes, although the expression of Rantes and IL-8 is induced by treatment with TNF-α or IFN-γ (Li et al 1996).

Interestingly Langerhans cells appear to downregulate MIP-1α expression rapidly on *in vitro* culture and maturation. This is somewhat surprising since the other main cytokine expressed by these cells, IL-1β, is gradually and massively upregulated during the *in vitro* culture and maturation of these cells into potent immunostimulatory dendritic cells (Heufler et al 1992). This is very different from what has been observed in macrophages where MIP-1α and IL-1β are both greatly upregulated on activation.

Langerhans cells are known to be immunologically naïve dendritic cells which undergo major changes in morphology, phenotype and function when they mature into potent

immunostimulatory cells. The stimulus for this alteration is either *in vivo* activation which results in their migration out of the epidermis or *in vitro* culture (Reviewed by Schuler in 'Epidermal Langerhans Cells': CRC Press 1991). Therefore the Langerhans cell expresses MIP-1α primarily when it is immunologically immature and paradoxically downregulates the expression of this protein when it is immunologically active. The role of this chemokine in the epidermis is unclear but these results suggest that the constitutive expression of this protein is only required when the epidermal Langerhans cell is immunologically naïve.

Interestingly the Langerhans cell has been previously postulated to play more than just an immunological role in the epidermis. Langerhans cells have in fact been implicated in the regulation of epidermal keratinocyte proliferation (Potten and Allen 1976). Could therefore Langerhans cell derived MIP-1α serve to function as a paracrine regulator of epidermal keratinocyte proliferation? Initial studies performed with the only available preparation of MIP-1α in our laboratory at the time, a partially purified protein derived from the transient transfection of COS 7 cells, have shown that this preparation of MIP-1α does reversibly inhibit the proliferation of clonogenic human epidermal keratinocytes (Parkinson et al 1993). Furthermore this activity is fully reversible with a specific polyclonal anti-MIP-1α antibody confirming that indeed this cytokine does not only inhibit the proliferation of primitive haematopoietic cells, but can also inhibit keratinocyte proliferation. These results appear to suggest that Langerhans cell derived MIP-1α may function as a physiological paracrine negative regulator of keratinocyte proliferation in the epidermis.

1.4 The Epidermis.

The epidermis is a stratified squamous epithelium. Structural and functional studies indicate that epithelial stem cells have many of the properties of haemopoietic stem cells. Epithelia, like the haemopoietic system, are constantly regenerating tissues. However epithelia, unlike haemopoiesis, have a high degree of visible structural order. Although many epithelial tissues differ in structure and function they mainly share a unifying theme in that they all undergo continual lifelong cell turnover which is characterised by specific patterns of proliferation within morphologically defined units (Potten and Morris 1988). The long life of these units, their patterns of proliferation and their response to irradiation or cytotoxic chemotherapy (clonal regeneration) all indicate that these units contain stem cells.

Epithelia are a diverse group of tissues which line most of the body's surfaces, cavities and tubes. Epithelial interfaces are involved in a wide range of activities such as protection, secretion and absorption. Supported by a basement membrane which separates the epithelium from the underlying connective tissue, epithelia consist of one or more layers of cells closely bound to one another by a variety of specialisations of the cell membrane. Epithelia are classified into two main categories depending on the number of cell layers. Simple epithelia comprise a single layer of cells while stratified epithelia are composed of more than one layer. Stratified squamous epithelia consist of a variable number of cell layers which undergo both morphological and functional transition from the cuboidal basal layer to the flattened surface layers. The basal cells undergo regular mitotic division giving rise to a succession of cells which are progressively pushed towards the free surface. During this migration to the surface these cells undergo differentiation. At the epithelial surface cells are being continually sloughed off and replaced from the deeper layers. There are basically two main types of stratified squamous epithelia: non-keratinising and keratinising. The epidermis is a stratified squamous keratinising epithelium. The differentiated epithelial cells of this tissue contain keratin, which is formed in those epithelia that cover exposed surfaces. Keratin is a tough fibrillar protein that gives the epidermis many of its special features. Fortunately the structural organisation of these two main types of stratified squamous epithelia are essentially similar. Unfortunately however the epidermis varies greatly in structure from site to site which considerably complicates epidermal cell-kinetic studies.

The epidermis is comprised mainly of keratinocytes which play a major role in providing the protective barrier of the skin. There are also a number of other cells in the epidermis which have their own specific function, such as the Langerhans cell, the melanocyte and the Merkel cell. These cell types will be discussed in turn.

1.4.1 The Keratinocyte.

Keratinocytes originate from the superficial ectoderm of the embryo. From a very early stage of development keratinocytes demonstrates a high degree of spatial and organisational specificity within the epidermis. These cells assemble into layers and in the post-natal skin vertically into columns. Interestingly the non-keratinocytes assume a non-random distribution among the keratinocytes. The epidermis can therefore be histologically subdivided into several layers or zones:

1) The basal layer or stratum basale
2) The prickle or spinous layer or stratum spinosum
3) The granular layer or stratum granulosum
4) The cornified keratin layer or stratum corneum.

1.4.1.1 The Basal Layer.

The basal layer cells are usually regarded as forming the proliferating compartment of the epidermis. Basal cells are distinguished by an intracellular cytoskeleton composed of a relatively dispersed, but extensive network of keratin filaments. These filaments are made up of a 1:1 ratio of two distinct keratin proteins K5 and K14 (Nelson and Sun 1983). Additional features of basal cells which are also found in the suprabasal spinous cells, are desmosomes, which interconnect the cells in a three dimensional lattice. Desmosomes contain desmogleins and desmocollins which are members of the cadherin family and connect to keratin filaments intracellularly (Reviewed by Fuchs 1993). Basal cells rest on the basement membrane and are in apposition superiorly with the spinous cells, border each other laterally, and have a definite polarity which can still be recognised in vitro (Stanley et al 1980). Their relationship with the underlying basement membrane, with which they interact by hemidesmosomes, is thought to be important not only in maintaining epithelial integrity but also in the regulation of epithelial cell differentiation (Watt F.M. et al 1988).

Although the basal layer was originally described as consisting of a homogeneous population of cells by Pinkus in 1927, it is now clear that this layer is made up of a heterogeneous population of cells structurally, functionally and also biochemically (Lavker et al 1982). Hibbs and Clark in 1959 were the first to note that basal cells appeared to be morphologically different in different parts of the epidermis. They observed that in human abdominal epidermis basal cells in the rete ridges were cuboidal in shape while as the basal cell approached the apices of the dermal papillae they became columnar in shape. They also noted that the basal cells at the apex of the rete ridges had less pronouced finger like projections into the dermis than basal cells at the apex of the dermal papilla. However their observations were ignored until cell kinetic studies strongly indicated that the basal layer was heterogeneous.

a) Basal keratinocyte heterogeneity. Where are the stem cells?

It is now thought that the basal layer comprises a mixture of cells with very different proliferative potentials. A large proportion of these basal cell have little or no proliferative potential and are poised to leave the basal layer and undergo the program of terminal differentiation. A large number of the remaining basal cells have some proliferative potential and although the precise position of the 'ultimate' epidermal stem cell remains uncertain there is no doubt that the basal layer contains clonogenic keratinocytes which are continuously proliferating (probably best described as transit amplifying or progenitor cells) and which are continuously replacing the cell loss in the layers above them. However Potten and Hendry (1973) have shown that when epidermis is severely damaged by irradiation only about 10% of the basal keratinocytes have sufficient proliferative potential to regenerate the epidermis by forming recognisable foci of new epidermis. Potten and Morris (1988) suggest that this 10% may be the stem cell population, although a further 50% of basal cells are also capable of cell division, the rest presumably being keratinocytes committed to terminal differentiation. However further studies suggest that these basal cells may not be the keratinocyte stem cell with the highest proliferative potential. For many years it has been observed that epidermal wounds in follicular epidermis can reepithelialize from the hair follicle, and studies by Al-Bawari and Potten (1976) demonstrated that after doses of irradiation that completely destroyed the interfollicular areas of epidermis, surviving cells

migrated from the follicle openings. Therefore in cases of extreme epidermal damage interfollicular epidermis can be regenerated by follicle derived cells. More recently studies have shown that the bulge region (or the upper intermediate part) of the follicle in both rats (Kobayashi et al 1993) and human scalp (Rochat et al 1994) contains a large number of epidermal cells with a very high proliferative potential (up to 130 doublings) suggesting that the ultimate epidermal stem cell may reside here. However the position of the epidermal stem cell in non-follicular epidermis and normal unwounded epidermis has not as yet been clearly defined.

Further evidence for the heterogeneity in basal keratinocyte proliferative potential comes from the *in-vitro* culture of keratinocytes. It has been demonstrated that when sheets of cultured epidermal keratinocytes are grafted onto a suitable recipient, they form epidermis that persists for considerable periods of time (Gallico et al 1984), suggesting that *in vitro* culture does not result in the loss of the stem cell compartment. However it has also been shown that only a small fraction of the basal keratinocytes cultured *in vitro* have a high proliferative potential. The *in vitro* assays of these cells reveal keratinocytes with very different proliferative potentials (Barrandon and Green 1987b). There are cells with a high proliferative potential which are known as holoclones. These colonies were made up of small very regular cells and can give rise to more holoclones or to paraclones which on the other hand have little proliferative potential and are at the end of their reproductive lifespan. The holoclone is thought to resemble the stem cell, with the paraclone being a mature progenitor. A mixed population of cells termed meroclones which appear to be transit amplifying or progenitor cells of varying maturity have also been described.

This *in vitro* data would be in keeping with the model proposed by Lavker et al in 1983 of a mixed population of basal keratinocytes functioning as follows:

Stem cells→Transit amplifying cells→Postmitotic cells

However it is not as yet clear how far this *in-vitro* data can be extended to the *in-vivo* situation. Also the successful transplantation studies of *in vitro* expanded keratinocytes do not necessarily indicate that the epidermal stem cell is still present in *in-vitro* culture, since there is some evidence to suggest that cells with only a relatively limited *in vitro* life

expectancy can be transplanted, forming healthy epidermis that can persist for several years (Gallico 1984). Moreover epidermal keratinocytes can only be passaged *in vitro* a limited number of times. Since stem cells are thought to last at least the lifespan of the individual, the holoclone which undergoes senescence after a limited number of passages may in some way be different from the stem cell. On the other hand *in vitro* culture may result in the loss of a cell's 'stemness' as a result of the stem cell being removed from its 'niche'. This niche, that is the stem cell's microenvironment, is thought to play a crucial role in preventing the cell from proliferation and/or differentiation (Potten 1981; Schofield 1983; Lajtha 1979), hence maintaining its "stemness". According to this niche theory once the stem cell is out of this *in-vivo* microenvironment and placed in tissue culture, where it encounters a very different milieu of growth factors and other stimuli that it does not usually encounter *in vivo*, the stem cell will exit the stem cell compartment and undergo active proliferation. Therefore the most primitive stem cells may not be maintained *in vitro.*

The slow cycling nature of the stem cell has also been used to demonstrate this heterogeneity of the epidermal basal layer. Bickenbach et al (1981) have shown that when newborn mice are labelled with a series of injections of tritiated thymidine, nearly all the basal keratinocytes are labelled initially. If the basal keratinocytes are a homogeneous population a 50% reduction in labelling at every round of division should occur. This would rapidly result in a low background level of labelling. However if the animals are examined several months after labelling a small proportion of heavily labelled cells can still be found. Although labelled basal non-keratinocytes have to be excluded (eg Langerhans cells which have a slow turnover in the epidermis and would also retain labelling), a population of label retaining basal keratinocytes is identified. Interestingly Morris (1986) has shown that when murine epidermis is treated with a labelled carcinogen, and the epidermis also exposed to tritiated thymidine, the basal layer label retaining cells are also found to be carcinogen retaining. It has been suggested that these label-retaining basal cells are the epidermal stem cells. On the other hand however it has also been shown that the bulge area of the hair follicle also contains large numbers of label retaining cells (Cotsarelis et al 1990). The significance of these label retaining cells with regards to interfollicular epidermis remains a matter for debate, although it appears clear that the hair follicle is the main repository of clonogenic keratinocytes (Rochat et al 1994). It has been proposed that under normal conditions the follicular stem

cells do not participate in the renewal of interfollicular epidermis. However each hair follicle constitutes a unique reserve of of clonogenic cells that can be recruited when the epidermis is damaged. Therefore under normal conditions clonogenic basal cells, the label-retaining cells, function as interfollicular epidermal stem cells (Rochat et al 1994). There is as yet little evidence to suggest that the formation of both hair and the epidermis is the property of a single follicular keratinocyte stem cell.

1b) The Basal Layer and Epidermal Structural Organisation: The EPU.

The basal layer which is clearly comprised of a heterogeneous population of keratinocytes has a definite pattern of organisation which appears to vary in complexity depending on the epidermal site. In certain sites such as the thin epidermis of the mouse ear or back, the epidermis is highly organised into vertical ordered columns of suprabasal keratinocytes and flattened terminally differentiated squames (Mckenzie 1969) which probably extends into the basal layer. This structural organisation has led to the proposal of the 'epidermal proliferative unit' (EPU) hypothesis by Potten (1974).

Since fixing, dehydration and embedding produce much distortion and limit the optical resolution, the ordered structure of epidermis was not appreciated until 1969 when specialised histological methods for analysing epidermis became available. The stratum corneum appears to consist of several flat and featureless layers when observed under normal H&E section. However when it is chemically swollen in 0.1M NaOH it reveals a remarkable degree of structural organisation (MacKenzie 1969; Christophers 1971.). The flattened keratinised cells are seen to be precisely stacked one above the other in ordered columns of cells. When viewed from above the squames appear as flat, hexagonal plates and are easily appreciated by scanning electron micrography, or by staining the cells using silver impregnation methods (Mackenzie and Linder 1973). These layers in thin mouse epidermis are particularly evident from the surface appearance of the epidermis after silver staining of sheets of epidermis or in scanning electron micrographs. These squames which appeared perfectly aligned into columns, can in the thin mouse back or ear epidermis, be projected back through the ten or so cell layers to the basal layer. These findings suggested that the epidermal columns could each be separate proliferative units (Potten et al 1974; 1976) termed 'Epidermal Proliferative Units' or EPUs. The EPU in mouse ear epidermis

contains approximately 20 cells, half of which are differentiating or differentiated, while the other half (~10) form a lower proliferating layer. Interestingly there is very little variation in the number of cells in this bottom layer per column/EPU. Of these 10 cells 3 or 4 appear to be arranged in a subunit consisting of a single Langerhans' cell and 2 or 3 basal cells in contact with it (Allen and Potten 1974). This subunit is surrounded by a ring of approximately 6 cells, where under steady state conditions most of the keratinocyte proliferation occurs. It is thought that each unit contains a single clonogenic cell (Potten and Hendry 1973). Studies using tritiated thymidine have shown that the central subunit in the EPU contains the slowly cycling label retaining cell while the peripheral ring of cells appear to be cycling faster than the central cells. These peripheral cells appear to be more mature cells (Christophers 1971) since upwards migration tends to occur at the EPU edges. The central label retaining cell in the EPU is unlikely to be the most primitive epidermal stem cell, since then approximately 10% of the basal layer cells, 5% of the total mouse ear epidermal cells, would be stem cells. In view of the probable heterogeneity of the stem cell and progenitor cell compartments it is likely that the central basal EPU cell may be a more mature stem cell, or like the day 12 CFU-S, an early transient amplifying cell.

The role of the immature dendritic Langerhans' cell in the central subunit of the EPU remains unclear. It has been postulated to be the EPU stem cell although this is now clearly incorrect (Morris 1985). The Langerhans' cell may however be involved in the regulation of the proliferation of the EPU clonogenic cell (Potten and Allen 1976).

Although our understanding of the proliferative organisation of the epidermis owes a lot to the EPU concept, unfortunately the epidermis is very variable in its organisational structure from site to site, between species and with age (Wright and Allison 1984). For example although primate epidermis shows a columnar pattern, there is a considerable degree of variation in the extent to which these columns interdigitate with each other. Also in some sites such as palmar and plantar epidermis which have a high turnover rate (including mouse footpad), there is no discernable EPU. In fact an EPU could not be demonstrated in oral mucosa, lip, nipple or vagina (Mackenzie 1973). On the other hand a number of investigators (Menton and Eisen 1971, Mackenzie 1981) have shown that there is a columnar pattern of organisation in human stratum corneum from the abdomen, scalp, chest and arm. However this could not be traced beneath the stratum granulosum. Also the overlap zone between contiguous squames

was more marked in human epidermis, although the stratum granulosum cells can exhibit a highly organised pattern (Hume and Potten 1983). Therefore in body sites where the epidermis is thicker, there is little structural evidence to support the existence of the EPU. Furthermore in experiments using chimeric animals, cells with markers that had originated from different embryos appeared to cross the EPU boundaries (Schmidt et al 1987) in mouse back epidermis. On the other hand however studies of the patterns of intercellular junction communication in both mouse (Kam et al 1986) and human epidermis (Salomon et al 1988) suggest that *in vivo* the epidermis is subdivided into many small communication compartments which are very similar both in size and organisation to the EPUs. Furthermore studies of retrovirally labelled keratinocytes, transduced *in vitro* and retransplanted back *to in vivo* sites, have demonstrated that these cells redevelop an ordered columnar structure with restriction of transduced, β-gal staining, cells to individual columnar units (Mackenzie et al 1997; Kolodka et al 1998). Therefore, although the concept of the EPU has been controversial, there is considerable evidence for its existence as an intrinsic feature of the epidermis.

Epidermal heterogeneity clearly complicates the study of epidermal stem cells. It is likely that such regional differences are intrinsic to the genetic program of the epidermal cells of the particular site. In palmar and sole epidermis, which are much thicker than most other body regions, the epidermis is thick at birth and contains large quantities of a specific keratin K9 which is only present in minute quantities at other epidermal sites (Knapp 1986). Also the transplantation of sole epidermis to the chest of a guinea-pig results in a graft that maintains the histologic features of its donor site (Billingham 1963). This suggests that this regional variability is intrinsic to the gene expression pattern of the cells (Miller et al 1993).

These regional differences in the epidermal cells' genetic program may explain the very different spatial order noted in monkey palm epithelium, which is highly organised into regular and alternating deep and shallow rete ridges (Lavker and Sun 1982 and 1983). They recognised two morphologically distinct populations of cells in the epidermal basal layer depending on the presence or absence of well-developed cellular projections extending deep into the papillary dermis, resulting in a highly convoluted epidermal-dermal junction. They described these cells as serrated and non-serrated cell respectively. The non-serrated cells were present in the basal layer of the deep rete ridges and appeared to be morphologically

primitive and slow cycling. They were heavily melanised and occupied a highly-protected site in the epidermis. These cells were thought to represent the palm epidermal stem cell population. They appeared to give rise to a population of rapidly proliferating cells located immediately above them in the suprabasal layers. The serrated cells were located in the basal layer of the shallow rete ridges and appeared to be transient amplifying epidermal cells that had ascended along the shoulder of the deep rete ridge.

Interestingly a separate group have also shown that receptors for epidermal growth factor (EGF) are primarily present on the basal cells in the deep rete ridges (Misumi 1991). Moreover β_1 integrin, which has been suggested to be a useful marker for the most primitive keratinocytes *in vitro* (Jones and Watt 1993), has also been shown to be expressed primarily in these same cells positioned in the deep rete ridges of human palm epidermis (Jones et al 1995) suggesting that this may be the site of epidermal stem cells in the non-follicular human palm skin.

c) The Integrins and Basal Cell Heterogeneity. A Keratinocyte Stem Cell Marker?

The integrins are a large family of widely expressed cell surface adhesion receptors (Hynes 1992). All integrins discovered to date are heterodimers of α and β subunits which are products of separate genes and are mutually interdependent for correct processing and surface expression. Integrins possess a generally conserved structure with a large extracellular domain, a transmembrane segment and usually a short intracellular C-terminal cytoplasmic domain. Integrins perform their functions primarily by interacting with components of the insoluble extracellular matrix or surface proteins on other cells. They serve as membrane-spanning molecular bridges between extracellular ligands - such as fibronectin, vitronectin, collagen, laminin - and intracellular cytoskeletal proteins. They are also important signalling molecules with the signal transmission occuring in two directions: extracellular cues are transmitted into the cell following integrin-ligand binding leading to events such as tyrosine phosphorylation, intracellular calcium fluxes, changes in the cytoskeleton, cell activation and differentiation. On the other hand cells can regulate the function of their surface integrins (Reviewed by Faull and Ginsberg 1995).

Studies have shown that as keratinocytes leave the basal layer they downregulate integrin function and expression (Adams and Watt 1990). This downregulation may be central to the

upward movement of basal cells committed to terminal differentiation. *In vitro* data suggests that during terminal differentiation, human epidermal keratinocytes lose their adhesiveness to fibronectin, laminin and collagen (types I and IV) because of the downregulation firstly of integrin function and then of cell surface integrin expression. This keratinocyte differentiation programme suggests that the more primitive epidermal basal cells have a special relationship with the underlying basement membrane. Interestingly in an *in vitro* differentiation model where human epidermal keratinocytes are induced to differentiate in a suspension of methylcellulose, the integrin ligand fibronectin is able to inhibit keratinocyte terminal differentiation by binding to the integrin $\alpha5\beta1$ receptor (Adams and Watt 1989). This suggests that the *in vivo* relationship of the undifferentiated keratinocyte with its 'niche' may be involved in the regulation of its differentiation.

Interestingly Jones and Watt (1993) have also shown that the proliferative potential of human epidermal keratinocytes *in vitro* bears a log linear relationship with the expression of $\beta1$ integrin. They have shown that cells with the highest colony forming efficiency are able to adhere most rapidly to type IV collagen, fibronectin or keratinocyte extracellular matrix and also have the highest expression of $\alpha2$, $\alpha3$, $\alpha5$ and $\beta1$ integrin, suggesting that these integrins may be markers for keratinocyte 'stemness' and may prove useful in determining the site of the most primitive epidermal cells. On the other hand keratinocytes with a limited capacity for cell division (between 1-5 rounds) adhered more slowly and expressed lower levels of the same integrins.

To examine the distribution of integrin expression immunohistochemical analyses of human skin from different sites have been performed (Jones, Harper and Watt 1995). The integrin-bright cells in human foreskin epidermis were found adjacent to the tip of the dermal papillae, while in the human palm the integrin-bright regions were in the tips of the deep rete ridges. Also in the scalp a cuff of integrin-bright cells was seen where the hair follicles opened onto the skin. These results indicate that the most primitive epidermal keratinocytes occupy these integrin-bright sites. However although this distribution in integrin expression in the basal layer is in keeping with the observation that the basal layer consists of a heterogeneous population of keratinocytes, nearly 40% of the basal cells were integrin bright, which suggests that not all integrin-bright basal cells are 'true' stem cells since it is thought that the proportion of basal cells that are stem cells is much lower than this. It is likely that the

integrin-bright population comprises not only the small stem cell pool but also a pool of transit amplifying cells. Therefore although β1 integrin is a surface marker of epidermal stem cells, it is not sufficient to define the stem cell pool exclusively. As for haemopoietic stem cells, a number of markers will probably be needed to separate out the epidermal stem cell pool.

Finally Jones et al (1995) have also shown that when primary foreskin keratinocytes are cultured on plastic dishes as sheets of cells that can be detached from the culture dishes with dispase, the keratinocytes form patches of integrin-bright cells. This suggests that integrin expression is an intrinsic feature of the clonogenic cell program and not dependent on the stem cell 'niche.' Interestingly, when the normal program of integrin expression in the epidermis is disturbed by the suprabasal overexpression of β1, α2β1, or α5β1 integrins in transgenic mice under the control of the involucrin promoter, epidermal homeostasis is upset with perturbed keratinocyte differentiation and epidermal hyperproliferation (Carroll, Romero and Watt 1995). The mechanism that results in this phenotype remains unclear. It has been postulated that the abnormal pattern of suprabasal integrin expression alters basal keratinocyte proliferation and differentiation.

1.4.1.2 The Stratum Spinosum.

As the basal keratinocytes move up into the suprabasal layers, they start flattening out and in histologic section appear to be joined to each other by 'spines' that protrude from the cell membrane. These spines correspond to bundles of keratin filaments which insert into the desmosomes of the adjacent cells. Spinous cells contain large and dense bundles of these differentiation-specific keratin filaments which form concentric rings around the nucleus. These cells devote much of their protein synthesising machinery to manufacturing these new keratins (keratin 1 and keratin 10) forming cytoskeletal filaments that aggregate into thin (tonofilaments) bundles (Eichner 1986). In addition spinous cells make glutamine and lysine rich envelope proteins which are deposited on the inner surface of the plasma membrane of each cell.

The stratum spinosum varies considerably in thickness, but in human skin it usually forms between four to eight layers, with the spinous cells in the upper layer being much more flattened than the suprabasal layer cells. In these uppermost cells new organelles, called lamellar granules can be identified, which are membrane-bound structures containing a

series of disk-like lipid bilayers which apart from providing the epidermis with its protective properties (Landmann et al 1988; Menon et al 1992), also promote the shedding of dead corneocytes (Williams et al 1991). These lamellar granules are clustered at the cell margins and contain the cell membrane coating lipids that will be released in the uppermost layer of the stratum granulosum.

1.4.1.3 The Stratum Granulosum.

The next epidermal layer has a characteristic appearance which gives it its name. The keratinocytes at this stage of their differentiation program contain irregularly shaped electron dense keratohyalin granules. In human epidermis this layer is usually two to three cell layers thick although it is thicker in the epidermis of the palm and the sole. The keratohyalin granules become progressively larger as the keratinocyte moves into the outermost granular layer and contain, in normal thin epithelium, the protein profilaggrin. Profilaggrin is a major component of keratohyalin and contains between ten and twelve tandemly repeated filaggrin units. Profilaggrin is processed into the intermediate filament-associated protein filaggrin by specific dephosphorylation and proteolysis. Filaggrin is a histidine rich basic protein which is involved in the bundling of tonofilaments into large macrofibrillar cables. This process of increased fibrillar packing is thought to enable the keratin filaments to survive the major cell destructive phase that will soon occur (Reviewed by Fuchs 1990). The keratinocytes in the granular layer also make the protein loricrin and a number of other structural proteins that will form major components of the cornified cell envelope. Cells in this layer have on the other hand stopped generating keratins and envelope proteins. As the granular cell reaches the stratum corneum it sheds the lipid filled lamellar granules. These coalesce outside the cell where they release their contents which seal the spaces between the granular and stratum corneum cells in lamellar sheets which are responsible for the barrier properties of the epidermis.

The granular layer is the last viable cell layer in the epidermis since the terminally differentiated stratum corneum is non-viable. The cells in the uppermost granular layer are in a state of preparation for the terminal differentiation program that results in keratinocyte programmed cell death or apoptosis. This complicated process, which is inadequately

understood, occurs rapidly over about 5-6 hours and results in the formation of the cornified layer.

1.4.1.4 Stratum Corneum.

The number of cell layers in the cornified layer depends on the skin site and is very variable. In the sole of the foot or the palms of the hands the stratum corneum is much thicker than in other body sites and may even be over a hundred layers thick. In regions where the skin is thin the number of cell layers is also variable (Leigh, Lane and Watt 1994). Cell size in the stratum corneum is also variable depending on the region, age and sex of the subject. Individual corneocytes also differ significantly in structure depending on their position in the stratum corneum relative to the skin surface and stratum granulosum.

The geometrical pattern of stacking of keratinocytes as they leave the suprabasal layers to enter the stratum corneum varies between skin site, species and age (Allen and Potten 1974; MacKenzie 1981) and has already been discussed (cf section on EPU). As these differentiating cells reach the stratum corneum they become permeable which allows the entry of extracellular calcium into the cell (Rice and Green 1979). This activates epidermal transglutaminase, which then catalyses the formation of peptide bonds that cross-link the keratinocyte envelope proteins into a rigid cage containing keratin macrofibrils. All vestiges of metabolic activity cease and the end result is flattened cellular skeletons which are fused together and suspended in a matrix of extracellular lipid. This barrier of dead terminally differentiated keratinocytes sealed together by lipid in a regularly arranged pattern forms an impermeable, insoluble protective barrier.

1.4.2 Non-Keratinocyte Epidermal Cells.

There are a number of other cell types in the epidermis apart from keratinocytes. Langerhans cells, melanocytes, Merkel cells and a number of other cells can also be found. These cells will now be discussed in turn.

1.4.2.1 The Melanocyte.

Melanocytes, unlike keratinocytes which are derived from the superficial ectoderm, are derived from the neural crest (Wright and Alison 1984). From this region undifferentiated melanoblasts migrate into the epidermis early in foetal development and take up a non-random distribution in the epidermis among the keratinocytes as a fixed cell population (Rawles 1947). Melanocytes are secretory dendritic cells and are predominantly found in the basal layer. They are easily recognised from the epidermal keratinocyte under the light microscope since they have a pale cytoplasm, lack keratin filaments and contain brown-black melanin pigment. Their unique characteristic is the presence of this pigment in melanosomes, which are small membrane bound structures in the cytoplasm that represent different stages in melanin granule formation. The number of melanosomes in a melanocyte is very variable and depends on the location of the melanocyte. Melanosomes are able to transfer the pigment in these melanin granules from their dendritic processes to keratinocytes, resulting in basal cell pigmentation. There is generally a fairly constant ratio of one melanocyte to thirty-six keratinocytes in the epidermis, and although this ratio may vary somewhat from site to site, there is little doubt that there is an intimate relationship between keratinocytes and melanocytes. The structural and functional significance of this distribution has been described in the epidermal-melanin unit (EMU) model.

1.4.2.2 The Merkel cell.

Merkel cells are neuroendocrine cells found in the epidermis, hair follicle and mucosal epithelium. Unlike both the melanocyte and the Langerhans' cell the Merkel cell probably originates in the epidermal ectoderm (Moll et al 1990) and in fact keratins have been identified in these cells. Like the melanocyte the Merkel cell is typically located in the basal layer. There are fewer Merkel cells than melanocytes or Langerhans' cells in the epidermis. In certain sites, such as the finger-pads, palms, feet, face and lips the Merkel cells are more prevalent. This is in keeping with the mechanosensory role of these cells. These cells are thought to be able to release neurotransmitters (Tachibana et al 1995) and are associated with intraepidermal unmyelinated nerve endings with which they appear to synapse. Merkel cells are thought to store these neurotransmitters in small intracellular membrane bound granules. These cells may also have a number of other functions and may have both

chemoattractive and trophic activities on peripheral nerve endings (Tachibana et al 1995). It has also been suggested that these cells may be involved in the regulation and maintenance of the keratinocyte population, perhaps by secreting paracrine factors that can modulate keratinocyte proliferation and differentiation (Hartshuhe 1989).

1.4.2.3 The γδ T-cell.

T-cells have been known to be present in epithelia for some time, however their function has been largely unknown. Although both αβ and γδ T-cells have been detected in human epidermis (Bos et al 1990), a number of studies have suggested that the majority of epidermal T-cells are γδ T-cells (Reviewed by Tigelaar and Lewis 1995). These cells form a dendritic network amongst basal layer keratinocytes and display striking T-cell receptor homogeneity. Nearly all epidermal γδ T-cells in normal adult mice, regardless of strain, express identical γδ T-cell receptors all using the same γ and γ gene coding segments and lack the diversity seen in other αβ and γδ T-cells (Asarnow et al 1988). Therefore unlike other T-cells, whose T-cell receptor heterogeneity permits the recognition of a broad array of antigens, γδ dendritic epidermal T-cell antigen recognition is very restricted and may be limited to one ligand. Interestingly such cells are found not only in the epidermis, but also in the epithelium of the tongue, intestinal tract, vagina and uterus (Itohara et al 1990).

The role of these cells is not fully understood (Reviewed by Boismenu and Havran 1998). It has been suggested that these cells may have an immune surveillance role, recognising a common self-antigen expressed by transformed, damaged or otherwise altered cells in the epidermal microenvironment (Asarnow et al 1988). The antigen in question has not as yet been characterised, although studies have shown that epidermal γδ T-cells are able to recognise physically/chemically stressed keratinocytes *in vitro* (Havran et al 1991). A number of studies have shown that dendritic epidermal T-cells are able to secrete a number of cytokines (Reviewed by Takashima and Bergstresser 1996) including IL-2, IL-3, IL-4 and IFN-γ. Furthermore these cells can be stimulated to secrete the keratinocyte growth factor KGF suggesting that γδ epidermal T-cells may be involved in the control of keratinocyte proliferation on epithelial wounding (Boismenu et al 1994). Interestingly T-cell receptor mutant mice lacking γδ T-cells have reduced intestinal epithelial cell turnover suggesting that these cells are involved in the regulation of the proliferation of intestinal epithelial cells

(Komano et al 1995). *In vitro* studies using an epidermal γδ T-cell cell line also suggest that these cells can be stimulated to secrete MIP-1α, MIP-1β, RANTES and lymphotactin (Boismenu et al 1996). The role of these molecules is not clear, although they too may be able to modulate the functions of nearby keratinocytes and Langerhans cells. However MIP-1α has not been detected in epidermal γδ T-cells *in vivo,* the only detectable source being Langerhans cells (see below). However epidermal keratinocytes do appear to secrete IL-7, which is both a mitogen and a survival factor for epidermal γδ T-cells. These and other studies suggest that a complex network of interactions exists between keratinocytes, γδ dendritic epidermal T-cells and Langerhans cells (Takashima and Bergstresser 1996).

1.4.2.4 The Langerhans Cell.

This cell was named after the German medical student Paul Langerhans who in 1868 described a population of intraepidermal dendritic cells in skin impregnated with gold chloride. This phenotype led him to postulate that these cells were nerve cells. It is now clear however that these dendritic cells are derived from the haemopoietic stem cell (Katz et al 1979; Frelinger et al 1979) and that there are a number of different Langerhans cell phenotypes (Nestle et al 1993). Dendritic cell progenitors are thought to originate in the bone marrow, enter the blood and seed non-lymphoid tissues such as the epidermis, where they develop into a stage of dendritic cell (sometimes referred to as immature dendritic cells) with optimal capabilities for antigen uptake, presentation and processing. Langerhans cell progenitors have been found both in bone marrow and circulating in peripheral blood (Inaba 1992a, 1992b) and large numbers of dendritic cells with a phenotype very similar to Langerhans cells can be cultured *in vitro* from both bone marrow and peripheral blood in the presence of cytokines such as GM-CSF and IL-4 (Romani et al 1994).

Under the light microscope the Langerhans cell is not easily identified in the epidermis. Their presence is sometimes suggested by their clear cytoplasm and their suprabasal position. The use of histochemical or immunohistochemical techniques (using anti-CD1a antibody) have made their identification easier. Langerhans cells do however have a characteristic ultrastructural appearance (Birbeck et al 1961). Under the electron microscope these cells possess a lobulated or indented nucleus, a clear cytoplasm and characteristically contain distinctive intracytoplasmic granules. The presence of these granules, now known as Birbeck

granules, has become established as one of the hallmarks of the Langerhans cell since these granules are not found in any other cell type. The function of Birbeck granules remains a matter for debate. Most of the granules in the Langerhans cell are found in the cell cytoplasm but some are found near or attached to the plasma membrane. This has led to two main lines of thought, one proposing that the Langerhans cell granule has a secretory role, the other suggesting an endocytic role (Reviewed by Schuler 1991). Despite some discord, it is clear that Langerhans cell granules can participate in the trafficking of molecules that enter the cell by receptor-specific endocytosis. The study of Langerhans cell protein secretion has been difficult since obtaining enough purified fresh Langerhans cells for these experiments involved the sacrifice of several hundred mice. However now that Langerhans cells can be cultured *in vitro* from bone marrow in large numbers these issues may be more easily addressed. Furthermore 'Langerhans cell like' cell lines have recently been isolated from the epidermis of newborn mice (Xu et al 1995a). These cell lines may be useful in these studies. However in view of the known functional and phenotypic heterogeneity of epidermal Langerhans cells and of these cell lines (Shibaki et al 1995; Xu et al 1995b), results from these studies may be only of limited value. The *in vitro* expansion of Langerhans cells from CD34 positive cells in the presence of the appropriate growth factors may also prove useful for these studies, although similar problems are likely to be encountered. Furthermore it has been observed that when Langerhans cells, freshly isolated and purified from the epidermis by immuno-magnetic panning, are cultured *in vitro* they alter their protein expression pattern which indicates that *in vitro* culture can alter the phenotype of these cells (Heufler et al 1992). Interestingly these studies have shown that freshly isolated Langerhans cells express IL-1β and MIP-1α mRNA. However on *in vitro* culture MIP-1α expression is downregulated while IL-1β is strongly upregulated. *In vitro* expansion and cell-line work must therefore be interpreted cautiously since the Langerhans cell's phenotype and activation state must be taken into account.

Epidermal development and the Langerhans cell.

Langerhans cells are thought to migrate into the epidermis early on in development and are distributed non-randomly in this tissue. Studies have shown that LC can be identified in human embryonic epidermis as early as 7-9 weeks estimated gestational age (Fujita et al

1991). These dendritic cells appear to be smaller and less dendritic than adult Langerhans cells, and phenotypically heterogeneous. Birbeck granules can easily be delineated by a specific anti-Birbeck Granule antibody by 10 weeks estimated gestational age. By the second trimester these cells resemble adult Langerhans cells both morphologically and phenotypically. Furthermore biopsies from different body sites in the second trimester indicate that the development of these epidermal dendritic cells undergoes regional differences during this time. Langerhans cell numbers in the palms and soles, which increase up to 12 weeks estimated gestational age, are seen to subsequently decrease and are rarely detected after 18 weeks. In other regions however, the Langerhans cell numbers are greater after 16 weeks estimated gestational age. Interestingly during this second trimester regional variations in epidermal development also become evident with the epidermis of the palms and soles showing earlier keratinisation with a thicker stratum corneum than elsewhere in the body (Holbrook et al 1980) but there is as yet no evidence to suggest that the two are related. In fact the mechanism that regulates foetal epidermal Langerhans cell numbers remains undefined.

Langerhans cell distribution.

In the adult these regional variations in Langerhans cell density persist. The number of Langerhans cells per mm^2 of skin surface varies from 460-1000/mm^2, the average adult human having approximately $2X10^9$ Langerhans cells in the skin (Reviewed by Schuler 1991). There are now several reported studies in the literature analysing Langerhans cell distribution. These studies also reveal that there is considerable inter-subject variability in Langerhans cell density at any given site.

The Langerhans Cell and the immune response.

There is no doubt that the Langerhans cell has immune functions during the inductive phase of immune reponses that originate in the epidermis. Langerhans cells function as potent accessory cells for the induction of primary T-cell dependent immune responses originating in the skin. It is also thought that Langerhans cells are immature precursors of lymphoid dendritic cells. Evidence for this comes from the in-vitro culture of freshly isolated epidermal Langerhans cells (Steinman et al 1995). In culture these cells increase in cell size and T-cell

immunostimulatory activity and there is extensive remodelling of the cell surface. Major histocompatiblity antigens class I and II products are upregulated as are ICAM-1 and LFA-3. This apparent phenotypic change is accompanied by a functional maturation as these cells become efficient at capturing and presenting foreign antigen to T-cells. After this these immunologically mature dendritic cells downregulate their antigen processing function and express their MHC-peptide complexes for several days. Interestingly cytokines such as GM-CSF and TNF-α are able to induce this dendritic cell maturation. The epidermal Langerhans cell therefore appears to be a phenotypically immature immune cell that can be induced to take up an immunologically mature phenotype on exposure to certain stimuli. Following antigen uptake these cells migrate from the skin through the dermis, where mature dendritic cells with some of the features of Langerhans cells have been observed, to the draining lymph nodes where they can initiate an immune response. Little is known about what happens to these dendritic cells after they have carried out their antigen presenting function in the lymph node. It has been suggested that after encountering antigen, mature dendritic cells can become targets for natural killer cell-mediated destruction (Shah et al 1986). It has also been proposed that these cells may undergo spontaneous apoptosis (Ludewig et al 1995).

There appears to be a considerable flux of dendritic cells in cutaneous afferent lymphatics This seems to argue against an origin from the less-abundant pool of epidermal Langerhans cells (Steinman et al 1995) particularly since a number of studies have shown that the turnover of the resident epidermal dendritic cells is slow in the steady state. In fact in the experiments that defined the haematopoietic origin of Langerhans cells (Katz et al 1979) it took 30-60 days for the donor bone marrow derived Langerhans cells to start replacing the original recipient cells. Similar findings have been observed by Krueger et al (1983) who found that the turnover of these epidermal dendritic cells took several months. Chen et al (1986) also observed prolonged survival of some dendritic cells in transplanted skin, finding donor skin derved Langerhans cells as late as six months after skin transplantation. Although proliferating Langerhans cells have been observed in the epidermis this is unlikely to explain the numerical discrepancy. The well recognised heterogeneity of this cell population may however in part account for these observations. There may be different pools of dendritic cells in the skin, some of which are long lived and others short lived (Steinman et al 1995).

Langerhans cells are therefore found not only in the epidermis, but also in the dermis, draining lymphatics and lymph nodes. They are also found in non-keratinised, stratified squamous epithelia like the conjunctiva, the corneal limbus, oral mucosa, tonsillar and oesophageal epithelium, vagina and uterine cervix. These cells are normally absent from other epithelia, but may settle there if squamous metaplasia has taken place (Reviewed by Schuler 1991). They are also found in the thymus. Considering the immune surveillance function of these cells as outposts of the immune system it is perhaps surprising that these cells are not more widely distributed. Dendritic cells have been observed in the lungs but these appear to be phenotypically distinct from epidermal Langerhans cells in that they lack the hallmark Birbeck granule. There are also CD1a positive cells in the stroma of the placenta and in the submucosa of the bladder, but their precise lineage remains to be characterised. It has been suggested that there may in fact be cells with many of the features of Langerhans cells present throughout the body, however this remains controversial.

The Langerhans cell and the regulation of keratinocyte proliferation.

It has also been suggested that the Langerhans cell is also involved in regulating the proliferation of keratinocytes in the epidermis. However although the hypothesis proposed is plausible, the evidence for the role of the Langerhans cell in keratinocyte proliferation has been circumstantial and must be interpreted with some caution. A number of questions remain. However it is thought that these cells must have other roles apart from immune surveillance (Schuler 1991). Moreover the epidermal Langerhans cell has been shown to constitutively express MIP-1α when it appears to be immunological quiescent (Heufler et al 1992; Matsue et al 1992; Parkinson et al 1993). The geographical location of the Langerhans cell in the EPU and the inhibitory profile of MIP-1α suggest that this multifunctional cytokine may not be functioning primarily as an inflammatory mediator in the epidermis. This chemokine may play a part in the network of regulatory cytokines that control the proliferation of primitive keratinocytes in the EPU (Parkinson et al 1993).

1.5. Regulators of Epidermal Keratinocyte Proliferation.

The decision of a clonogenic keratinocyte to enter the cell-cycle from quiescence is controlled by a number of positive and negative regulatory growth factors. A number of keratinocyte mitogens have been identified. These include members of the Epidermal Growth Factor (EGF) family such as Transforming Growth Factor-α (TGF-α), amphiregulin and EGF itself as well as the Fibroblast Growth Factor homologue Keratinocyte Growth Factor (KGF) and Interkeukin 6 (IL-6). A number of negative regulators have also been characterised and these include TGF-β, TNF-α, and the Epidermal Pentapeptide (EPP). These growth factors and their role in epidermal keratinocyte proliferation will now be discussed in turn.

1.5.1 Positive Regulators.

1.5.1.1 The Epidermal Growth Factor (EGF) Family.

The EGF family of growth factors is characterised by a conserved amino-acid sequence including six essential cysteine residues that form three conserved disulphide bonds (Reviewed by Todaro et al 1990). This structurally conserved domain is characteristically cleaved from a larger membrane-bound precursor polypeptide and is then released into the surrounding medium. The soluble growth factor can then function to regulate cell proliferation in an autocrine or paracrine manner. A number of EGF-like molecules have been descibed, however only EGF, TGF-α and amphiregulin will be discussed here.

a) Transforming Growth Factor-α (TGF-α).

TGF-α was initially discovered as a factor produced by cells transformed by sarcoma viruses and by various human tumour cells. This factor binds to the EGF receptor and is able to convey a transient transformed phenotype to normal cells in culture (DeLarco et al 1979; Todaro et al 1980). A 50 amino-acid polypeptide, human TGF-α is highly homologous to human EGF, and like EGF the primary translation product of the TGF-α gene exists as a transmembrane glycoprotein. The mature TGF-α molecule is proteolytically derived from this transmembrane precursor (Teixido et al 1987; Gentry et al 1987). TGF-α is expressed in a wide variety of tissues including the epidermis. Further study has shown that TGF-α is expressed by epidermal keratinocytes and functions as a keratinocyte autocrine regulator

(Coffey et al 1987). TGF-α also promotes keratinocyte migration *in vitro* and it has been suggested that this migration is essential for the maintenance of keratinocyte colony growth (Barrandon and Green 1987). This cytokine functions in the epidermis by activating epidermal growth factor receptors (EGFRs) on the surface of basal keratinocytes and is more potent than EGF.

Elevated levels of TGF-α have been associated with a variety of hyperproliferative skin diseases, such as psoriasis and cancer (Gottlieb et al 1988; Elder et al 1989; Derynck et al 1987). The expression of TGF-α in tumours is often accompanied by enhanced synthesis of the EGF/TGF-α receptor (Gottlieb et al 1988). In squamous cell carcinomas elevated levels of both TGF-α and EGF receptors have been identified (Ozanne et al 1986). Further evidence for a direct role for TGF-α and the EGF receptor in the pathogenesis of hyperproliferative epidermal disorders comes from transgenic mice overexpressing TGF-α in the basal layer of the epidermis under the control of the keratin 14 promoter (Vassar and Fuchs 1991). These mice have a wrinkled and flaky skin, with a markedly thickened epidermis and bromodeoxyuridine labelling indicates that there is approximately a two-fold increase in the number of DNA synthesising cells in the mitotically active basal layer. These mice also have a pronounced granular layer in the epidermis. Interestingly these mice do not appear to be at an increased risk of squamous cell carcinoma although skin papillomas are common at sites of injury or mechanical abrasion, as well as on TPA treatment. These results suggest that although TGF-α is a very potent keratinocyte mitogen, TGF-α dysregulation alone is not sufficient to induce carcinogenesis.

However TGF-α does appear to be crucial to the determination of normal skin architecture since TGF-α knockout mice have a deranged epidermal structure (Mann et al 1993; Luetteke et al 1993). Although mice homozygous for a disrupted TGF-α gene are healthy and fertile, with a normal thickness epidermis and apparently normal keratinocyte maturation, their coat is abnormally wavy with pronounced waviness of the whiskers and hair and there is considerable disarray, disorientation and misalignment of the hair follicles. These mice also have corneal abnormalities with corneal scarring and superficial opacity as well as reduced eyeball size and open eyelids at birth. These results therefore indicate that TGF-α plays a central role in determining the architecture of the epidermis and the corneal epithelium. Since surprisingly loss of TGF-α does not appear to influence epidermal keratinocyte proliferation

or differentiation it is likely that the loss of this cytokine can be compensated for by recruitment of a related molecule encoded by a different gene. This is likely since a number of different ligands are known to be able to compete with TGF-α for binding to the EGF receptor. Confirmation of this hypothesis comes from EGF-receptor (EGF-R) knockout mice which have impaired epithelial development in a number of organs including the epidermis (Miettinen et al 1995). These mice die soon after birth and have abnormally thin skin which is often almost transparent. This skin is very fragile and contains a significantly decreased number of hair follicles which are disorientated and which did not often penetrate the epidermis. These mice have a much more severe phenotype than TGF-α knockouts which indicates that a number of EGF-R ligands are involved in binding to the EGF-R in the epidermis.

b) Epidermal Growth Factor (EGF).

EGF was first isolated from the submammary gland of the male mouse as a protein that accelerated incisor tooth eruption and eyelid opening in the new-born animal (Cohen 1962). It was then shown to be a a potent mitogen for epidermal keratinocytes (Cohen 1975). Further studies have shown that EGF is mitogenic for a number of other cell types in culture (Reviewed by Prigent and Lemoine 1992). A 53 amino-acid peptide, it is derived from a 1217 amino-acid precursor that is initially expressed as a transmembrane molecule. Proteolytic cleavage of this precursor generates the mature EGF protein. EGF is not detected at all in foetal life (Popliker et al 1987). In the adult apart from the curiously high concentrations found in the submaxillary gland, in urine and in milk, EGF only appears to be expressed at easily measurable levels in the adult kidney and by the Brunner's glands of the small intestine (Rall et al 1985). *In vivo,* in the epidermis, EGF is therefore likely to play a secondary role to TGF-α. Like TGF-α, EGF also functions by activating the 170kDa tyrosine kinase EGF receptor which is expressed by almost all cell types but most abundantly by epithelial cells (Green et al 1984). Like TGF-α, EGF is a potent mitogen for epidermal keratinocytes. EGF also stimulates the migration of these cells from the centre of keratinocyte colonies *in vitro*, and is used in the routine culture of these cells *in vitro* (Rheinwald and Green 1977).

c) Amphiregulin.

Amphiregulin is a heparin-binding member of the EGF receptor ligand family and was initially discovered as a secreted product of a human breast adenocarcinoma cell line MCF-7 treated with TPA (Schoyab et al 1988). The truncated form consists of 78 amino-acids and the carboxyl terminal half of this cytokine is highly homologous to EGF. Amphiregulin is a bifunctional growth regulator, stimulating the proliferation of normal epithelial cells but inhibiting the growth of a variety of human tumour cells. Amphiregulin competes with EGF in binding to the EGF receptor, and can substitute for EGF and/or TGF-α in stimulating the proliferation of both human and murine keratinocytes (Shoyab et al 1989). Recent studies suggest that like TGF-α, amphiregulin may be involved in the pathogenesis of a number of hyperproliferative disorders (Piepkorn et al 1996).

d) Heparin-binding EGF-like Growth Factor (HB-EGF).

HB-EGF is a new member of the EGF family and was initially purified from the conditioned media of the U-937 macrophage-like cell line (Higashiyama et al 1991). Like TGF-α it is expressed by human keratinocytes and functions as an autocrine factor for these cells (Hashimoto et al 1994). It is therefore clear that there are a number of EGF-R ligands that can function as mitogens, implying a degree of functional redundancy already seen in the haemopoietic system. This helps explain why knockout mice deficient in only one of these keratinocyte mitogens do not have as severe a phenotype as the EGF-R knockouts.

Non-EGF family mitogens.

1.5.1.2 Keratinocyte Growth Factor (KGF): A FGF-like Keratinocyte Mitogen.

Keratinocyte Growth Factor (KGF) is a member of the fibroblast growth factor (FGF) family. Also known as fibroblast growth factor 7 (FGF 7), it is made by dermal fibroblasts and is a paracrine growth factor for epidermal keratinocytes (Rubin et al 1989; Finch et al 1989). In culture KGF is a more potent growth factor than the autocrine growth factor TGF-α. *In vivo* KGF mRNA transcripts can be detected in the dermis of normal and wounded skin (Finch et al 1989). Furthermore a high affinity tyrosine kinase receptor for KGF (KGFR) has been identified on cultured keratinocytes (Miki et al 1991, 1992), although KGFR is not present on dermal fibroblasts. KGFR has been shown to be a splice variant of the FGF receptor-2 (FGFR2), and can bind both KGF and acidic FGF (aFGF) which is also a keratinocyte mitogen. Since KGF is the most potent keratinocyte mitogen known, and since the level of KGF expression has been shown to increase by as much as 160 fold (Werner et al 1992) upon skin injury, it is likely that this paracrine factor secreted by dermal fibroblasts plays an important role *in vivo*. Direct evidence for this *in vivo* role comes from transgenic mouse studies. Transgenic mice were generated in which KGF expression was driven by the keratin 14 promoter to elicit autocrine production of the factor to the basal layer of the epidermis (Guo et al 1993). These mice exhibited a gross increase in epidermal thickness accompanied by alterations in epidermal growth and differentiation, with marked suppression of hair follicle morphogenesis and suppression of adipogenesis. The few follicles that did develop however developed normally. As with the TGF-α transgenic animals, KGF transgenic mice developed no skin papillomas.

These results suggest that KGF can function as a potent mitogen *in vivo*. To further investigate the function of KGF *in vivo* KGF knockout mice have been generated (Guo et al 1996). The fur of these mice develops a rough, greasy and matted appearance with time indicating that the maintenance of a smooth and healthy hair coat is dependent on KGF. However further study on these mice has surprisingly shown that these mice develop no abnormalities in epidermal growth or wound healing. Furthermore TGF-α and KGF double knockout mice also have no evidence of any abnormality in epidermal proliferation or wound healing (Guo et al 1996). This is perhaps not surprising since both EGF and aFGF can activate EGFR and KGFR respectively, and therefore still be able to control keratinocyte

proliferation in the absence of KGF. Therefore the KGF knockout mice have shed little light on the *in vivo* role of KGF in the regulation of epidermal proliferation and in wound healing.

To try to elucidate this a dominant negative KGF receptor transgene has been constructed and overexpressed in the basal cells of mouse epidermis under the control of the Keratin 14 promoter (Werner et al 1994). It has been previously shown that mutated FGF receptors that lack kinase activity block FGF receptor signalling by forming non-functioning hetero-dimers with wild-type receptors on ligand binding (Ueno et al 1991). One would therefore expect the epidermal basal cells of these transgenic mice to be unable to signal through the KGFR. Interestingly the skin of these mice appears to be macroscopically normal. However histological analysis has revealed that these mice develop epidermal atrophy, with flattened epidermal basal cells which have pyknotic nuclei after six weeks of life, being normal at birth. BRDU analysis has confirmed that there is a reduced steady state proliferation rate in the epidermis, suggesting that loss of KGFR signalling perturbs the homeostatic mechanisms involved in maintaining keratinocyte proliferation. In addition these mice exhibit abnormal hair follicle morphology as well as reduced hair follicle numbers. Furthermore the dermis of these mice appears to be altered as well, with thickening of the dermis characterised by replacement of the normal dermal adipose tissue by connective tissue. This may be an indirect result of the loss of epidermal keratinocyte KGF reponsiveness, since the transgene does not appear to be expressed in the dermis. Furthermore wound-healing and re-epithelialisation are impaired in these trasgenic mice indicating that signalling through the KGFR may be important for normal keratinocyte migration and proliferation in wound-healing. However although the results of the K14 driven, dominant negative KGFR transgenics appear quite conclusive it has to be pointed out that this dominant negative receptor can complex with all FGFRs. Furthermore this transgene product may have downstream effects that go beyond its direct effect on the FGFR pathway. Therefore from this data alone, it is difficult to understand the precise role of KGF in the epidermis. However it certainly appears likely that the FGFR tyrosine kinase pathway is involved in the regulation of epidermal proliferation and wound-healing.

1.5.1.3 Other Positive Regulators.

A number of other cytokines have been shown to enhance the growth of keratinocytes *in vitro*. These include IL-6 (Grossman et al 1989), and the chemokine IL-8 (Sticherling 1989). Both of these cytokines have been identified in psoriatic plaques and have therefore been implicated in the pathogenesis of this hyperproliferative skin disorder. IL-6 is also interesting in that the overexpression of IL-6 in the basal cells of stratified squamous epithelia of transgenic mice under the control of the keratin 14 promoter results surprisingly in mice that have a thickened stratum corneum. The mechanisms by which increased IL-6 overexpression in the basal layer of the epidermis causes a thicker stratum corneum are not fully understood. However these mice are also smaller than normal and have retarded hair growth (Turksen et al 1992). The *in vivo* role of the CXC chemokine IL-8 in the epidermis remains undefined. It is however interesting to note that epidermal keratinocytes can express IL-8, suggesting a possible autocrine role for this chemokine.

It is therefore clear that there are a number of positive regulators of keratinocyte proliferation. On the other hand fewer negative regulators have been identified, perhaps because these molecules are generally more difficult to characterise in *in vitro* assays.

1.5.2 Negative Regulators of Keratinocyte Proliferation.

A number of negative regulators of keratinocyte proliferation have been described. These factors induce the withdrawal of the epidermal keratinocyte from the cell-cycle into a quiescent or Go state. This withdrawal from the cell-cycle may be important not only to regulate keratinocyte stem cell/progenitor cell numbers, but also for the irreversible commitment of a keratinocyte to terminally differentiate (Fuchs et al 1993). The most extensively studied negative regulators of epidermal cell proliferation are the TGF-βs.

1.5.2.1 TGF-β.

Although the prototype of the TGF-β family, TGF-β1, was originally defined as a factor which stimulated the proliferation of normal rat kidney fibroblasts in soft agar (Roberts et al 1981), further work has demonstrated that this family of cytokines has a wide number of different biological activities. In fact studies have shown that TGF-β is a negative regulator of haemopoietic stem cell proliferation (see section 1.2.3.5) as well as a potent negative

regulator of keratinocyte proliferation *in vitro* and *in vivo* (Shipley et al 1986). TGF-β1 has been shown to cause a marked reduction in [^{3}H] thymidine labelling in cultured human keratinocyte progenitors as well as inducing growth arrest in keratinocyte precursors in serum free medium. The majority of the keratinocyte progenitors cultured in the presence of TGF-β arrest in the G_1 phase of the cell-cycle (Shipley et al 1986; Coffey et al 1988a). Since this growth inhibition is reversible this effect is not due to cytotoxicity. Furthermore TGF-β has been shown to induce terminal differentiation in normal human epithelial cells (Masui et al 1986), although this is not a general mechanism of action for this cytokine since this does not occur in either cultured human foreskin keratinocytes nor in mouse keratinocyte cell lines (Shipley et al 1986; Coffey et al 1988b). Interestingly keratinocyte progenitors have been shown to secrete bioactive TGF-β, as well as having specific TGF-β surface receptors, suggesting that TGF-β functions in an autocrine fashion in primitive keratinocytes. In fact both anti-TGF-β antibody and the TGF-β latency associated peptide (LAP) are able to increase the proliferation rate of epidermal keratinocyte progenitors (Bottinger et al 1996). It is therefore clear that TGF-β is involved in the autocrine regulation of keratinocyte proliferation regulation *in vitro*. TGF-β is also a potent regulator of keratinocyte proliferation *in vivo*. Transgenic mice overexpressing TGF-β1 in the epidermal suprabasal layer under the control of the keratin K1 promoter results in animals that have a taut shiny skin (Sellheyer et al 1993). Epidermal hyperproliferation has also been observed in TGF-β1 knockout mice, although the skin in these mice is of normal thickness suggesting that other members of the TGF-β family may take over the role of TGF-β1 in its absence (Glick et al 1993). Furthermore the keratinocytes of these mice have a much higher rate of malignant progression both in vivo and in vitro (Glick et al 1994a and 1994b). Moreover the expression of a dominant negative type II TGF-β receptor in the epidermis of transgenic mice blocks TGF-β mediated growth inhibition and results in markedly hyperplastic and hyperkeratotic epidermis (Wang et al 1997). These results demonstrate that TGF-β is a physiological negative regulator of epidermal keratinocyte progenitors *in vivo*.

1.5.2.2 TNF-α.

TNF-α has been shown to inhibit the proliferation of epidermal keratinocytes (Pillai et al 1989; Vieira et al 1996). Moreover keratinocytes and keratinocyte cell lines can secrete TNF-α, although unstimulated keratinocytes express only low levels of TNF-α mRNA and bioactive protein (Kock et al 1990). Since TNF-α has been reported to induce programmed cell death (Reinartz et al 1996), the inhibitory activity of TNF-α on keratinocytes may be related to an induction of apoptosis in these cells. However this keratoinhibitory activity may be reversible. This suggests that TNF-α is a negative regulator of keratinocyte proliferation, although it can also stimulate keratinocyte differentiation (Pillai et al 1989).

Further work has shown that keratinocytes express receptors for TNF-α and that the signal transduction cascades activated by this receptor regulate the expression of the cell-cycle regulatory proteins Cyclin A, Cyclin B and cdc2 as well as the phosphorylation state of the retinoblastoma protein Rb (Vieira et al 1996). These downstream events probably result in the growth arrest observed in these cells and indicate that TNF-α is a negative regulator of keratinocyte proliferation *in vitro*. However TNFα may play any one of a number of different potential roles in the epidermis since it has been shown to be bioactive *in vitro* not only on keratinocytes, but also on melanocytes and Langerhans cells (Reviewed by Wakefield et al 1991).

1.5.2.3 Epidermal pentapeptide (EPP).

The pentapeptide (pyroGlu-Glu-Asp-Ser-Gly) was initially purified from mouse epidermis as an inhibitor of epidermal keratinocyte proliferation (Elgjo et al 1986). Further work has demonstrated that synthetically synthesised EPP also inhibits epidermal keratinocyte proliferation both *in vivo* and *in vitro*. This molecule is therefore a potential physiological regulator of keratinocyte regulation. Furthermore interestingly EPP has also been shown to inhibit murine hair growth *in vivo* as well as inhibit the incorporation of 3H thymidine into murine anagen hair follicles *in vitro* (Paus et al 1991). Therefore this pentapeptide may be involved in the control of not only epidermal keratinocyte proliferation but also hair follicle growth. There is however as yet little evidence to suggest a physiological role for this molecule.

1.5.2.4 MIP-1α

Recent studies have shown that the chemokine MIP-1α is constitutively expressed in the epidermis by the epidermal Langerhans cell (Matsue et al 1992; Heufler et al 1992; Parkinson et al 1993). It has also been shown that an impure preparation of COS 7 cell derived MIP-1α is a potent and reversible inhibitor of clonogenic keratinocyte proliferation *in vitro* (Parkinson et al 1993). However further investigation has shown that the bacterial recombinant form of MIP-1α does not inhibit keratinocyte proliferation although it does inhibit the CFU-A and CFU-S haemopoietic assays. On the other hand the activity of the COS 7 cell derived kerato-inhibitory form is reversible by a specific polyclonal antibody to bacterial recombinant MIP-1α (R&D systems). This has strongly suggested that MIP-1α does inhibit clonogenic epidermal keratinocyte proliferation, although the discrepancy in the activity of these two forms of MIP-1α has not been explained.

Aims of Thesis.

A number of studies have shown that the chemokine MIP-1α is a multi-functional cytokine, with a variety of different activities. This is not surprising since it is now clear that many cytokines are multi-functional. TGF-β for example has potent anti-inflammatory properties, while also being a powerful inhibitor of the proliferation of a wide variety of cell types, such as primitive haemopoietic and keratinocyte progenitors. MIP-1α has been shown to have a number of inflammatory properties. However it can also inhibit the proliferation of primitive haemopoietic cells as well as protect these multipotent cells from the toxicity of cytoreductive chemotherapy.

MIP-1α has also been shown to be expressed by a number of different cell types. Most of these cell types require some form of stimulation or activation to express this cytokine. However this chemokine is constitutively expressed in the epidermis by the epidermal Langerhans cell. The role of this molecule in the epidermis is unclear. However a number of observations suggest that this cytokine may not be behaving simply as an inflammatory mediator in normal epidermis. Since MIP-1α is known to be a negative regulator of haemopoietic progenitor cell proliferation, it is possible that MIP-1α may function in a similar manner on keratinocytes. Preliminary results have shown that when derived from the transient transfection of COS 7 cells, MIP-1α reversibly inhibits keratinocyte proliferation. However the pure bacterial recombinant molecule is inactive on keratinocytes, although it is active on the haemopoietic assay. The aim of this project was therefore to investigate the reason for this discrepancy, and to try and elucidate the potential role of MIP-1α in the epidermis.

Materials

2.1. Tissue Culture Supplies

Beatson Institute Central Services	Sterile PBS Sterile glassware and pipettes Sterile PE Sterile double distilled water Penicillin(7.5mg/ml) Streptomycin(10mg/ml)
Amicon (USA)	Centriprep 10 spin columns
Becton Dickinson UK Ltd	Tissue culture plates (35,60,90 mm) Falcon tubes (15ml and 50ml)
Clonetics (USA)	Bovine pituitary extract Epidermal growth Factor Insulin Hydrocortisone Amphotericin Gentamycin Keratinocyte growth medium
Costar	6 well plates Cell scrapers
DIFCO Laboratories (Michigan, USA)	Bactoagar
Gibco Life Technologies (Paisley, UK)	Special Liquid Medium Keratinocyte SFM Dulbecco's modified Eagles medium Foetal calf serum MEM alpha medium 200mM glutamine Sodium bicarbonate Trypsin
Nunc (Roskilde, Denmark)	Tissue culture flasks Nunc cryotubes
Gelman Sciences (UK)	Sterile acrodiscs syringe filters (0.2mm and 0.45mm)
Genetics Institute, Mass. (USA)	Human MIP-1α
R&D Systems (UK)	Bacterial recombinant Murine and human MIP-1α Polyclonal neutralising antibody to muMIP-1α,TNF-α Recombinant Latency Associated Peptide *(Neutralizes TGF-β1,2 and 3)* Non-aggregating MIP-1α mutants

SIGMA Chemical Co. (Poole, UK)	Ampicillin Agarose bound chymotrypsin Plasmin Thrombin Cathepsin G Donor herd horse serum Bovine serum albumin (fraction V) Heparin sulphate Heparan sulphate Chondroitin sulphate DEAE dextran Chloroquine
Worthington Biochemical (UK)	Trypsin

2.2. Bacterial hosts and media

Beatson Institute Central Services	L-broth Sterile glassware
Beta Labs (UK)	Yeast extract
Bibby-Sterillin Ltd (UK)	Sterilin 9cm bacteriological plates
Difco labs (Detroit, USA)	Agar Bactotryptone Bactoagar
Gibco (Paisley, Scotland)	E.Coli host strain DH5α Ampicillin

2.3. Molecular Biology

Beatson Institute Oligo Service	Oligonucleotides
Bethesda Research Laboratories Gibco(UK)	DNA molecular weight markers DNA mass ladder Agarose and low melting point agarose Restriction endonucleases with buffer concentrates Taq DNA polymerase, 10X PCR buffer, $MgCl_2$. T4 DNA ligase and ligase buffer Calf Intestinal Alkaline Phosphatase
Pharmacia	dNTPs (100mM set)
Qiagen Inc.	Qiagen plasmid preparation kits Qiaquick DNA purification kits

2.4. **Protein Biochemistry**

Amersham International (UK)	ECL Western blotting detection reagents Rainbow molecular weight markers
BDH Chemicals Ltd. (Poole, UK)	Acrylamide bis-acrylamide All chemicals other than those listed below.
Boehringer-Mannheim UK (Lewes, UK)	
Dow Chemical Company (UK)	Saranwrap
Du Pont NEN	Iodine 125*
Eastman Kodak Co. (Rochester, USA)	X-OMAT AR X-ray film DUP-1 duplicating film X-ray cassettes
Millipore	Immobilon P
Pharmacia	FPLC LCC-500 system Heparin Sepharose CL-6B Blue Sepharose Sephadex G-25 Resource 1 ml reverse phase column Hi-Trap desalting columns
Pierce & Warriner (UK)	GF5 excellulose desalting columns Iodogen reagent
R&D Systems (Abingdon, UK)	Quantikine transforming growth factor β-1 immunoassay
Schleicher & Schuell (Dassel, Germany)	Nitrocellulose membranes
Severn Biotech Ltd. (Kidderminster, UK)	Design-a-Gel 40% (w/v) acrylamide, 2% (w/v) bis-acrylamide solution.
Sigma Immunochemicals (UK)	Anti-goat IgG Anti-rabbit IgG (Both horseradish peroxidase conjugate) Sodium orthovanadate PMSF Aprotonin Trifluoro-acetic acid (TFA) Tricine Silver Nitrate
Technical Photo Systems (Cumbernauld, UK)	Fuji Medical RX X-ray film
Whatmann International Ltd. (Maidstone, UK)	3MM filter paper

2.5. Chemicals

SIGMA chemical Co. (Poole, UK)	Bromophenol blue Dithiothreitol (DTT) MOPS TEMED Triton-X 100 FURA-2 AM
James Burroughs Ltd. (Witham, UK)	Ethanol
GIBCO BRL (Paisley, UK)	TRIzol reagent
Fisons Scientific Equipment (Loughborough, UK)	Formaldehyde (38% w/v) Propan-2-ol Glycerol HPLC grade acetonitrile Anhydrous sodium carbonate Magnesium chloride Sodium chloride
Fluka Chemika-Biochemica AG (Switzerland)	Formamide
Taab Laboratories and Equipment Ltd	Glutaraldehyde

2.6. Solutions

2.6.1 Tissue Culture

MEM Alpha Stock (Gibco)	Dissolve 5 litre pack in water, add 50ml MEMx100 vitamins (Gibco) and 100mg gentamycin sulphate. Make up to 1500ml with water and filter sterilise.	
MEM Alpha x 2 (for 100ml)	MEM alpha stock	21ml
	L-Glutamine (200mM)	1ml
	Sodium Bicarbonate (7.5%)	3ml
	Donor Horse Serum	25ml
	(For MEM Alpha x 1, add equal volume of distilled water or agar)	
RPMI 1640	RPMI 10x stock	100ml
	Distilled water	860ml
	L-Glutamine (200mM)	10ml
	Sodium Bicarbonate (7.5%)	1.5ml
L-broth (L-Amp broth)	Yeast extract	5g
	Bactotryptone	10g
	NaCl	10g
	Ampicillin (50mg/ml)	

2.6.2 Agarose Gel Electrophoresis, Northern Blotting and Hybridisation

10 x TE (for 1 litre)	Tris/HCl pH8	12.1g
	EDTA	3.7g
	Water to 1 litre	

2.6.3 SDS-PAGE Polyacrylamide Gel Electrophoresis

Resolving Gels

17.5% Polyacrylamide gel	Water	3 ml
	Acrylamide mix (30%)	11.6ml
	1.5M Tris (pH 8.8)	5.0 ml
	10% SDS	0.2ml
	10% ammonium persulphate	0.2ml
	TEMED	0.01ml
15% Polyacrylamide gel	Water	4.6ml
	Acrylamide mix (30%)	10 ml
	1.5M Tris (pH 8.8)	5 ml
	10% SDS	0.2ml
	10% ammonium persulphate	0.2ml
	TEMED	0.01ml
5% Stacking gel	Water	6.8ml
	Acrylamide mix (30%)	1.7ml
	1.0M Tris (pH 6.8)	1.25ml
	10% SDS	0.1ml
	10% ammonium persulphate	0.1ml
	TEMED	0.01ml
10 x SDS/DTT	20% SDS	0.1ml
	1M DTT	0.1ml
	1M Tris (pH 6.8)	30µl
	Complete to 1ml with water	
1x SDS/PAGE buffer	Glycine	28.8g
	Tris.HCl	6.05g
	SDS	2.0g
	up to 2l with dH_2O	

Methods

2.7. Cell Culture

2.7.1 Derivation and culture of human epidermal keratinocytes.

Normal human epidermal keratinocytes were obtained from human foreskins, obtained after paediatric therapeutic circumcisions, from Yorkhill children's hospital, Glasgow. The tissue was washed in PBS, cut into thin strips and trypsinised overnight at 4°C using 0.125% (w/v) trypsin, 0.01% EDTA in PBS. Epidermal cells were then separated from the underlying dermis by scraping a scalpel along the epidermal layer. Cells were centrifuged at 1000rpm for 5 minutes and resuspended in a single cell suspension in growth medium.

Human epidermal keratinocytes (HEKS) were grown in serum free keratinocyte medium (KGM) supplemented with bovine pituitary extract (50μg/ml), recombinant epidermal growth factor (5ng/ml), recombinant insulin (5μg/ml) and hydrocortisone (0.5 μg/ml). Penicillin (37.5μg/ml) and streptomycin (10 μg/ml) were also added to inhibit bacterial infection. All cells were incubated in a moist atmosphere at 37°C and gassed with air containing 5% (v/v) carbon dioxide. The medium was changed every 2-3 days.

Cells were passaged at subconfluence by firstly rinsing with PBS and then removal from flasks or plates with trypsin (trypsin 0.1%(w/v), EDTA 0.01%(w/v) in PBS) for approximately 5-10 minutes at room temperature. Trypsin was inactivated by adding ten volumes of serum-containing medium and removed by centrifugation at 1000rpm for 10 minutes at 4°C.The supernatant was then removed and the cells resuspended in fresh growth medium and replated if required.

The cells were harvested after approximately 20 population doublings. Stocks of HEKs were kept frozen in liquid nitrogen as follows. The cells were resuspended at a concentration of 10^6/ml in ice-cold special liquid medium (SLM; Gibco) containing 10% serum (as above) plus 10%(v/v) dimethylsulphoxide (DMSO). The cells were then aliquoted into cryotubes and the ampoules wrapped in cotton wool and placed in a plastic box. The ampoules were initially placed at -70°C for 24 hours and then stored in liquid nitrogen. The cotton wool insulation ensures gradual cooling of the cells and enhances cell viability on thawing.

Cells were thawed by transferring the ampoule directly from liquid nitrogen to water at 37°C. Once thawed the cells were added to ten volumes of prewarmed growth medium, centrifuged, resuspended in fresh growth medium and plated as required.

2.7.2 HEK clonogenic assays (KGM assays).

HEKs were seeded at 10^4 cells per 35mm culture well in six well plates in the appropriate keratinocyte growth medium with or without the protein preparation being assayed. The medium was removed after 72 hours and the adherent cells refed with the same KGM/preparation under assay. After 5 days the colonies were rinsed with PBS, and then fixed in 10% (v/v) formaldehyde in PBS. The cells were stained with 10% Giemsa/PBS for 10 minutes. The stain was then washed off with water and the plates left to dry before being scored under a low power microscope.

2.7.3 Cell lines.

All cell lines were obtained from the frozen stocks of the Beatson Institute and were maintained at 37°C in a dry atmosphere of 5% CO_2 in air. The COS 7 cell line, which is an SV40 transformed African Green Monkey kidney cell line (Gluzman et al 1981) and the Chinese Hamster Ovary epithelial cell line (CHO) originated by Puck (1955) were maintained in special liquid medium (SLM) containing 10% (v/v) foetal calf serum (FCS) and 4mM glutamine.

2.7.3.1 CHO and COS 7 cell lines.

These cell lines were seeded at 10^5/ ml of medium and allowed to reach near-confluence. The growth medium was then removed, the cells rinsed with warm PBS, and then trypsinised by incubating with 0.25% trypsin in PBS for five minutes at room temperature. The cells were then resuspended in fresh medium containing serum and quantitated using a haemocytometer. They were then centrifuged at 1000rpm for 5 minutes. The cells were resuspended in fresh medium at a concentration of 10^5/ ml.

2.8. Mice

All mice were housed within the animal facility of the Beatson Institute. Female mice, aged between 8-12 weeks were used for all the experiments. For the in-vitro CFU-A assay bone marrow from the femurs of the mouse strain B6D2F1 was used, whereas for the derivation of bone marrow macrophages, femoral bone marrow from strain CD1 mice or MIP-1α knockout mice (strain C57B1, which were a generous gift of Dr Don Cook (Dept. of Pathology, University of North Carolina at Chapelhill, NC.) were used.

2.9. Harvesting of bone marrow

Bone marrow was obtained from femora of mice. Mice were killed either by CO_2 asphyxiation or cervical dislocation. The femurs were then carefully removed using surgical scissors, and stripped of excess muscle using a tissue soaked in ethanol. Both ends of the femur were removed with scissors and the bone marrow plug expelled by flushing with 2mls of SLM through a 21 gauge needle. The cells were re-suspended thoroughly by vigorous pipetting using an automatic pipette aid. The cells were then washed in PBS and nucleated cells quantitated using a haemocytometer. Approximately $1.5x10^7$ cells were routinely obtained from each femur. Cells were then resuspended at the appropriate concentration and in the appropriate medium for their application (see below).

2.10. The In Vitro CFU-A Assay.

Bone marrow cells were obtained as described above. For the detection of primitive progenitor cells, CFU-A assays were carried out. $2x10^4$ total bone marrow cells in 4mls of supplemented α-modified MEM containing 20% DHS and 0.3% Bacto agar (Difco, USA) were seeded on top of an underlayer of the same medium containing 0.6% Bacto agar / 10% L929 CM / 10% AF1-19T CM in 45mm Petri dishes (Sterilin). Cultures were incubated at 37^oC in a fully humidified atmosphere of 10% CO_2, 5% O_2 and 85% N_2 for 11 days. This semi solid agar culture gave rise to the formation of macroscopic colonies, which have been previously shown to consist of mature haemopoietic lineages (Pragnell et al 1988; Lorrimore et al 1990). Although colonies smaller than 2mm in size do appear in the CFU-A assay, only colonies greater than 2mm were scored since it has been previously demonstrated that these larger colonies are derived from primitive haemopoietic cells. For all experimental

procedures involving this assay, a "direct addition" protocol was adopted whereby putative inhibitory cytokines are assessed for their potential effects on CFU-A colony formation by directly adding various concentrations to the bottom layer of agar (Graham et al 1992).

2.11. Recombinant DNA techniques.

2.11.1 Host cells.

Library efficient competent *E.coli,* strain DH5α (Gibco) was used. DH5αs and their derivatives were grown at 37°C shaking at 150 rpm in a new Brunswick G25 shaker in Terrific broth or L-broth liquid medium, or on inverted 1.5% (w/v) agar L-broth plates also at 37°C, in both cases supplemented with the appropriate antibiotics. Ampicillin was used at a final concentration of 50μg/ml.

2.11.2. Transformation of bacterial hosts.

Competent cells were thawed slowly at 4°C, and 100μl aliquots put into precooled 15ml 2059 falcon tubes. An appropriate amount of plasmid DNA or ligation mix (<10ng) was pipetted onto the cells and then mixed by gently tapping the tube, and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 45 seconds, cooled on ice for two minutes and then 0.9mls of L-broth added. This was incubated for 1hour at 37° C shaking at 225rpm in a New Brunswick G25 shaker, to allow time for the expression of the ampicillin resistance gene. 100-200μls of this infected broth was then spread onto 1.5% (w/v) agar/ L-broth plates supplemented with the appropriate antibiotic. The plates were allowed to dry for five minutes, then inverted and incubated overnight to allow colony formation. To identify colonies containing recombinant plasmid in which the lac Z gene of the vector had been disrupted, blue/white selection on X-gal was often employed. Prior to spreading the cells, 40μls of a 20μg/ml solution of X-gal, mixed with 160mls of L-broth, was spread onto the plate and left for one hour to permit full absorption into the agar. White colonies will contain a non functional lacZ gene.

2.11.3 Bacterial glycerol stocks.

Host strains and their derivatives containing the plasmids of interest, were stored as glycerol stocks for future retrieval. Stationary cultures in liquid medium were mixed with an equal volume of a 30 % (v/v) glycerol/ L-broth solution, cooled on ice, then frozen at -70°C. Cells were retrieved using a sterilised tungsten loop.

2.11.4. Preparation, Manipulation and purification of plasmid DNA.

a) Preparation of plasmid DNA.

Plasmids were prepared using the commercially available Qiagen plasmid kit (Qiagen Inc. USA) as follows. Bacterial pellets were resuspended in the recommended volume (depending on the scale of the plasmid preparation) of resuspension buffer (100mg/ml RNase A, 50 mM Tris/HCl, 10mM EDTA, pH 8.0). The suspension was transferred to non-glass Oakridge centrifuge tubes (Nalgene) and the appropriate volume of lysis buffer (200mM NaOH, 1% SDS) was added. The solutions were then mixed by gentle inversion and incubated at room temperature for 5 minutes. The required volume of neutralisation buffer (3M KAc, pH 5.5) was added, mixed and incubated on ice for 15 minutes. Tubes were then spun at $4^{O}C$ for 30 minutes at 10000 rpm in a Sorvall RC-5B superspeed centrifuge containing SS-34 rotors. The supernatant was then poured through a double layered gauze swab to remove particulate material. To purify the DNA, a QIAGEN-tip column (size 100, 500, or 2500) was equilibrated with equilibration buffer (750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7). The filtered supernatant was then applied to the column, and the column washed with wash buffer (1M NaCl, 50mM MOPS, 15% ethanol, pH 7). DNA was then eluted using the recommended volume of elution buffer (1.25M NaCl, 50mM Tris/HCl, 15% ethanol, pH 8.5) for that particular column. DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 10000 rpm at $4^{O}C$ for 30 minutes as described above. The DNA pellet was washed with 5ml of ice-cold 70% ethanol and following recentrifugation with 70% ethanol at room temperature. The pellet was then air dried for 5 minutes, redissolved in a suitable volume of sterile distilled water and stored at -20°C.

b) Measurement of nucleic acid concentration by spectrophotometry.

The absorbance (A) at 260 nm and 280 nm of DNA and RNA solutions was measured in a 500μl quartz cuvette, using TE, distilled water or TE/0.1% (w/v)SDS as a blank where appropriate. An A_{260} of 1 unit is equivalent to 50μg/ml of plasmid or genomic DNA, and 40 μg/ml of RNA or single-stranded DNA. The OD_{260}/OD_{280} ratio gives an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have an OD_{260}/OD_{280} ratio of 1.8 and 2.0 respectively. If theOD_{260}/OD_{280} ratio was significantly lower than the above, this was due to contaminants, which were removed by phenol, chloroform/isoamyl alcohol extraction and ethanol precipitation before remeasurement of the OD_{260}/OD_{280} ratio. When the concentration of the nucleic acid in the sample was too high ($A_{260}>1$), the sample was diluted in distilled water and the OD_{260}/OD_{280} ratio remeasured.

c) Restriction digests.

Restriction digests were carried out in small volumes buffered using concentrated solutions available with the restriction enzymes from the supplier (GIBCO). Small quantities of plasmid DNA (2μgs) were digested in a total volume of 20μls using 5-10 units of enzyme per μg of DNA, depending on the enzyme used and the number of sites present. Larger preparative digests were carried out in larger volumes. For double digests, suppliers' information was consulted and the appropriate buffer used.

d) Agarose gel electrophoresis.

DNA fragments were resolved on non-denaturing agarose gels and visualised by ethidium bromide staining. In general 1% (w/v) agarose gels were used, but smaller fragments (100-500bp) were separated on 2% agarose gels. Gel mixes containing the appropriate amount of agarose in 1X TAE buffer (50X TAE buffer is 2M Tris, 50mM EDTA, 57.1 ml/L glacial acetic acid) were heated in a microwave to dissolve the agarose and then cooled to approximately 60°C. Ethidium bromide was then added to make up a final concentration of 5μg/ml, and the gel mix was then poured into the appropriate gel caster. Gels were allowed to set at room temperature and then installed into the electrophoresis tank in 1X TAE buffer. Samples containing one-tenth volume of gel-loading buffer were loaded and separated at a constant

voltage ~100V. Molecular weight standards used include the bacteriophage λ (Hind III-digested) and the 1kb ladder (Gibco). After the DNA had migrated the required distance, the DNA was visualized by UV fluorescence of the ethidium bromide on a UV transilluminator. A Polaroid photograph of the illuminated DNA was usually taken.

e) Purification of DNA fragments from agarose gels.

DNA fragments were purified from regular TAE agarose gels using the QIAGEN QIAquick gel extraction kit. The DNA fragment was excised with a sterile scalpel from the agarose gel on the UV transilluminator. The DNA fragment was then weighed in an eppendorf tube and three volumes/ weight equivalent of the supplied buffer QX1 was added to one volume of gel. The reaction was incubated at 50°C for 10 minutes. To help dissolve the gel the reaction was mixed by inverting the eppendorf tube several times during the reaction. 10μls of 3M sodium acetate pH 5.0 was then added and the sample loaded onto the QIAquick column which was placed in a 2ml collection tube. The reaction was centrifuged in a microfuge at 13000rpm for 60 seconds and the flow-through fraction discarded from the collection tube. The QIAquick column was returned to the same collection tube. The DNA was then washed with 0.75mls of the supplied buffer PE and centrifuged again for 60 seconds. The wash flow-through was discarded and the column recentrifuged for an additional minute. This extra spin removes residual wash buffer. The QIAquick column was then placed in a clean 1.5 mls eppendorf tube and the DNA eluted by adding 50μls 10mM Tris-HCl pH 8.5 to the middle of the column membrane and cenrifuging again at 13000 rpm for one minute. The DNA was then stored at -20°C.

f) Ligation of DNA fragments into plasmids.

Both plasmid and potential DNA insert were digested and purified as described above. The plasmid was dephosphorylated at its termini to prevent religation, by including two units of calf intestinal alkaline phosphatase in the restriction enzyme digest reaction. Approximately 20 ng of dephosphorylated plasmid DNA was then added to the following reaction mix in a final volume of 20μls with water : 4-fold molar excess of the potential DNA insert, 4μls of 5X ligation reaction buffer (0.25 M Tris HCl (pH7.6), 50mM $MgCl_2$, 5mM dATP, 5mM dithiothreitol, 25% (w/v) polyethylene glycol-8000), and 1μl of T4 DNA ligase (1U/μl). The

reaction was incubated at 16 °C in the water-bath in the cold-room for 16-24 hours. 1-3µls of the ligation reaction mix was then diluted 5 fold in TE pH7.5 (10mM Tris-HCl and Na_2 EDTA) and used to transform competent cells as described.

2.11.5. Engineering DNA fragments by the polymerase chain reaction using plasmid DNA as template: Site directed mutagenesis by overlap extension.

a) Design and synthesis of oligonucleotides.

For each mutant two single-stranded oligonucleotide (oligo) primers were synthesised and purified as descibed below. The oligos were 20-40 bases in length and were designed such that they were complimentary to opposite strands and opposite ends of the DNA sequence of interest. The oligos contained the required restriction enzyme sequence 5' to the sequence complimentary to the template. This allowed subcloning of the required PCR-generated DNA fragment into the appropriate restriction sites of the chosen plasmid vector. The 3' ends of the designed oligos was either a G or a C base if possible in order to enhance priming from the oligo.

Oligonucleotides were synthesised at the Beatson Institute on an Applied Biosystems model 381A DNA synthesiser according to the manufacturers' instructions. The DNA was immobilised on a column and eluted from the column in 29% ammonia (v/v), by passing the ammonia continuously through the column for three five minute periods separated by 20 minute intervals. The resulting DNA-ammonia solution was sealed in a glass vial and incubated overnight at 55°C. The DNA was then precipitated in 2.5 volumes of ethanol at -20°C by adding ammonium acetate to a final concentration of 0.1MNH_4Ac. and then centrifuged at 13000 rpm in a microfuge. The oligos were resuspended in 100µls water and precipitated by the addition of 2.5 volumes of ethanol and then sodium acetate pH 5.2 to a final concentration of 0.1M Na Ac. This was mixed by vortexing and cooled to -70°C for one hour, and the nucleic acid pelleted by centrifugation for 30 minutes at 13000 rpm at 4°C in a microfuge. The DNA was then washed in 70% (v/v) ethanol,freeze-dried and dissolved in distilled water. After quantitation by spectrophotometry the oligos were diluted to a 10µM 10X stock and stored at -20°C until required.

b) Polymerase chain reactions (PCRs).

PCRs were carried out using Taq polymerase as specified by the manufacturer (Perkin Elmer). The PCR reactions were performed in a total volume of 50μls and the template used was circular plasmid DNA containing the sequence of interest, diluted to a final concentration of 10^{-7} to 10^{-9}g/ μl. The primers were used at a concentration of 10nM. The reaction contained 5μls of 10X Taq polymerase buffer (provided with the enzyme by the manufacturers); 2μls from 5mM stock solutions of each of dATP, dTTP, dGTP, and dCTP; 3 μls of 25mM $MgCl_2$; the primers and template and finally distilled water to 50μls. The reaction was overlayed with 50μls of paraffin. The Taq was added just prior to starting the PCR reaction. The reaction mixture was subjected to 25 to 30 cycles of denaturation (1 minute, 94°C), annealing (90 seconds, 50°C) and extension (5 minutes, 72°C) using a Perkin -Elmer-Cetus DNA thermal cycler 480. Reactions were incubated for 10 minutes at 72°C after the final cycle, then gradually cooled to 4°C. The PCR reaction products were analysed by agarose gel electrophoresis and the required DNA band purified from the agarose gel by the QIAquick method already described.

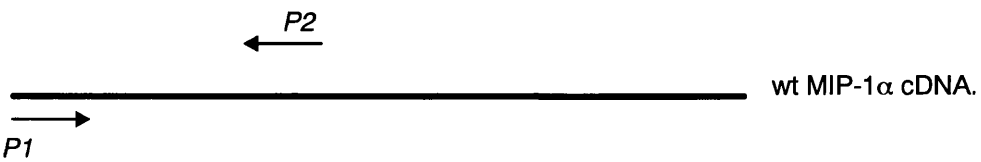
c) PCR mediated site-directed mutagenesis of plasmid DNA sequences: gene splicing by overlap extension.

The polymerase chain reaction was used to generate specific mutations in the murine MIP-1α cDNA. To generate each mutant four specific primers were used (Primers P1, P2, P3, P4.). Primers P1 and P4 were complimentary to approximately 30 bases of opposite DNA strands, at the opposite termini of the murine MIP-1α cDNA. Primers P2 and P3 contained sequences complimentary to opposite strands of the DNA site to be mutated and primed in opposite directions. These two primers, P2 and P3, contained the point mutation in the overlapping region. Primers P2 and P3 were both designed to have essentially three sections with the central section having the deletion mutation of interest. The flanking ends (around this mutation) of the primers P2 and P3 were complimentary such that these primers contained 30-36 complimentary bases.

To generate each mutant required three separate PCR reactions. The first two PCR reactions involved primers P1 and P2, and primers P3 and P4 respectively in separate reactions with the wildtype cDNA as template and the PCR reaction conditions already

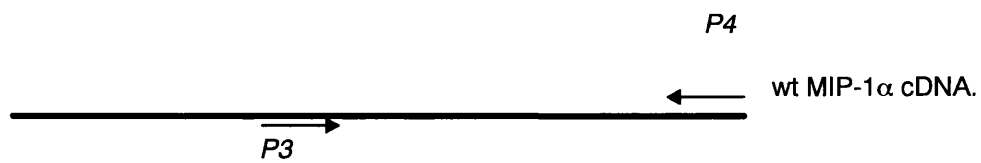
described. These two reactions generated overlapping double-stranded DNA fragments containing the required point-mutation in the overlapping region. These two double stranded DNA fragments were then purified by gel extraction and used as a template for a final PCR reaction with the end primers P1 and P4. This resulted in the full-length mutated cDNA of interest (see diagram below). Approximately 100ng of each DNA fragment was used as template.

PCR reaction 1



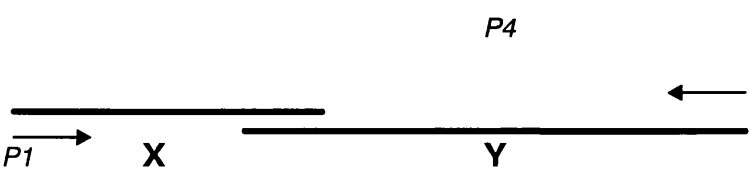
This PCR reaction generates the N-terminal end of the mutant cDNA (X).

PCR reaction 2



PCR reaction 2 generates the C-terminal end of the mutant cDNA (Y). Since the designed primers P2 and P3 both span the mutated site, the generated cDNAs X and Y overlap. These two cDNAs were therefore purified and used together as a template to yield the cDNA of the MIP-1α mutant by a third PCR reaction.

PCR reaction 3



The full-length mutated cDNAs were gel purified and ligated into the plasmid pMT_x and cloned as already described. Miniplasmid preparations of the clones obtained were then performed and their cDNA inserts sequenced by the dideoxy-mediated chain termination method described below to eliminate PCR-generated artifact and identify the required cDNA

clones. The required clones were then utilised to generate the required mutant MIP-1α cDNA plasmids. These plasmids were then used to generate the required protein mutants by the transient transfection of COS 7 cells using DEAE dextran.

Wildtype MIP-1α cDNA sequence.

-23 -20 -10

Met Lys Val Ser Thr Thr Ala Leu Ala Val Leu Leu Cys Thr Met Thr Leu

ATG AAG GTC TCC ACC ACT GCC CTT GCT GTT CTT CTC TGT ACC ATG ACA CTC

+1 +10

Cys Asn Gln Val Phe Ser Ala Pro Tyr Gly Ala Asp Thr Pro Thr Ala Cys

TGC AAC CAA GTC TTC TCA GCG CCA TAT GGA GCT GAC ACC CCG ACT GCC TGC

+20

Cys Phe Ser Tyr Ser Arg Lys Ileu Pro Arg Gly Phe Ileu Val Asp Tyr Phe

TGC TTC TCC TAC AGC CGG AAG ATT CCA CGC CAA TTC ATC GTT GAC TAT TTT

+30 +40

Glu Thr Ser Ser Leu Cys Ser Gln Pro Gly Val Ile Phe Leu Thr Lys Arg

GAA ACC AGC AGC CTT TGC TCC CAG CCA GGT GTC ATT TTC CTG ACT AAG AGA

+50 +60

Asn Arg Gln Ile Cys Ala Asp Ser Lys Glu Thr Trp Val Gln Glu Tyr Ile

AAC CGG CAG ATC TGC GCT GAC TCC AAA GAG ACC TGG GTC CAA GAA TAC ATC

+69

Thr Asp Leu Glu Leu Asn Ala End

ACT GAC CTG GAA CTG AAT GCC TGA

Primers used:

P1 **CCG CGC CTC GAG ATG AAG GTC TCC ACC ACT GCC**

P4 **CCG CGG AAT TCA GGC ATT CAG TTC CAG GTC AGT**

- 1 **AAC CAA GTC TTC TCA CCA TAT GGA GCT GAC**

GTC AGC TCC ATA TGG TGA GAA GAC TTG GTT

- 2 **AAC CAA GTC TTC TCA TAT GGA GCT GAC ACC**

GGT GTC AGC TCC ATA TGA GAA GAC TTG GTT

-3 **AAC CAA GTC TTC TCA GGA GCT GAC ACC CCG**

CGG GGT GTC AGC TCC TGA GAA GAC TTG GTT

-4 **AAC CAA GTC TTC TCA GCT GAC ACC CCG ACT**

AGT CGG GGT GTC AGC TGA GAA GAC TTG GTT

-5 **AAC CAA GTC TTC TCA GAC ACC CCG ACT GCC**

GGC AGT CGG GGT GTC TGA GAA GAC TTG GTT

-6 **AAC CAA GTC TTC TCA ACC CCG ACT GCC TGC**

GCA GGC AGT CGG GGT TGA GAA GAC TTG GTT

-7 **AAC CAA GTC TTC TCA CCG ACT GCC TGC TGC**

GCA GCA GGC AGT CGG TGA GAA GAC TTG GTT

-8 **AAC CAA GTC TTC TCA ACT GCC TGC TGC TTC**

GAA GCA GCA GGC AGT TGA GAA GAC TTG GTT

-9 **AAC CAA GTC TTC TCA GCC TGC TGC TTC TCC**

GGA GAA GCA GCA GGC TGA GAA GAC TTG GTT

Amino-terminally truncated MIP-1α mutants.

APYGADTPTACC.....	wildtype
PYGADTPTACC.....	-1
YGADTPTACC.....	-2
GADTPTACC.....	-3
ADTPTACC.....	-4
DTPTACC.....	-5
TPTACC.....	-6
PTACC.....	-7
TACC.....	-8
ACC.....	-9

Mutants -1 and -7 were not secreted by COS 7 cells, while mutant -9 appeared to be poorly secreted. A number of studies have previously shown that the amino-acid proline (P) at position +1 does not allow signal peptidase cleavage (Von Heijne 1986; Nothwehr and Gordon 1990). Therefore primers were synthesised to generate -1 and -7 mutant cDNAs with proline to alanine substitutions.

AYGADTPTACC...	-1 P→A
ATACC...	-7 P→A

Primers used:

-1P→A	AAC CAA GTC TTC TCA GCA TAT GGA GCT GAC
	GTC AGC TCC ATA TGC TGA GAA GAC TTG GTT
-7P→A	AAC CAA GTC TTC TCA GCG ACT GCC TGC TGC
	GCA GCA GGC AGT CGC TGA GAA GAC TTG GTT

Again these cDNAs were ligated into the plasmid pMTx, and the mutant protein was expressed by the transient dextran transfection of COS 7 cells. The -1 P→A mutant and the -7 P→A mutant were both secreted as detailed in the Western blot below.

2.11.6. Sequencing of plasmid DNA.

Sequencing was carried out on the Applied Biosystems ABI373 DNA sequencing system by Robert Mcfarlane. The recommended Applied Biosystems Taq Terminator sequencing protocol for this system was used (PRISM Ready Reaction Dyedeoxy Terminator Cycle Kit). This kit produces terminated fragments from unlabelled primers extended by AmpliTaq DNA polymerase. Each dideoxynucleotide is labelled with a different fluorescent dye so that one can carry out the four base reactions simultaneously in one tube and electrophorese them together in one gel lane. Approximately 1μg of double-stranded plasmid DNA was mixed with 3.2 pmoles of the sequencing primer and the volume was made up to 10.5μls with high grade deionised water. 9.5μls of the Applied Biosystems premix was then mixed with the above and the reaction performed in a Perkin Elmer thermal cycler 9600. The thermal cycler was initially heated to 96°C and the reaction hot-started. The thermal cycler program used involved a 15 second period at 96°C, followed by 1 second at 50°C and then 4 minutes at 60°C. 25 cycles were performed and at the end of this the mix was soaked at 4°C.

To remove the unincorporated dye terminators two phenol/chloroform extractions were performed. To do this 80μls of high grade deionised water were first added to the reaction mix, followed by 100μls of phenol/chloroform/water (68:18:14). The mixture was vortexed for 60 seconds and then microfuged at 13000rpm. The aqueous (upper) phase was separated from the lower (organic) phase. The organic phase was discarded and the aqueous phase retained. The extraction was repeated with another 100μls of phenol/chloroform/water. 15μls of 2M sodium acetate pH 4.5 was then added to the final aqueous phase and the dye labelled DNA precipitated using 2.5 volumes of ethanol. The reaction was mixed and left to stand on dry ice for 15 minutes. The DNA was then spun down in a microfuge (13000rpm for 15 minutes) and the supernatant carefully removed. The DNA pellet was washed with 500μls 70% ethanol and reprecipitated by another spin (13000rpm for 15 minutes). The supernatant was carefully removed and the pellet dried under vacuum for 5 minutes. An appropriate volume of loading buffer was added to the pellet and this was then heated at 92°C for two

minutes and then put on ice. The sample was then loaded onto a 6% acrylamide/urea gel, and the dideoxyterminated DNA run. The Applied Biosystems ABI 373 hardware and software is then able to generate sequence data by simultaneous multicolour dye detection. The sequences generated were then checked manually and corrected if necessary.

2.12. Introduction of recombinant vectors into mammalian cells.

2.12.1 Transfection mediated by DEAE-Dextran.

COS 7 cells were transfected using DEAE dextran as a facilitator to introduce plasmid DNA into the cells. The method used was that described in "Molecular Cloning - A laboratory Manual" (Sambrook, Fritsch and Maniatis 1990). Twenty-four hours before transfection, exponentially growing COS 7 cells were harvested by trypsinisation and seeded onto 10 cm dishes at $5X10^5$ cells/dish. The cells were incubated for 24 hours at 37°C in a 5% CO_2 incubator. The plates were then aspirated and the cells rinsed twice with serum and glutamine free SLM (Gibco). 4 mls of DEAE dextan DNA media was then added to each plate and the cells were incubated for 12-14 hours with shaking at 37°C in 5% CO_2.

DEAE dextran DNA media was made up by adding 5mls of DEAE dextran (2.5mg/ml), 5mls of 1M Tris pH7.3, 0.5mls of glutamine (200mM), 0.5 mls of streptomycin (10,000U/ml) and 0.67 mls of penicillin (750μg/ml) and 38.3 mls of SLM (GIBCO). This solution was filter sterilised using a 0.2μ filter (Gelman Sciences) and the plasmid DNA of interest was then added to a concentration of 0.1 to 1μg/ml.

The cells were then rinsed with prewarmed (37°C) serum and glutamine free SLM and then incubated for 5 hours in 5mls of SLM containing 10% heat inactivated foetal calf serum containing 0.1mM chloroquine (the foetal calf serum was heat inactivated by treatment at 55°C for 30 minutes). After the chloroquine treatment, which is thought to enhance transfection efficiency, each dish was rinsed once with serum-free SLM and then incubated in 10 mls of SLM containing heat inactivated foetal calf serum for 24 hours. The COS 7cells were then rinsed once with serum free SLM and incubated for 72hours in 4 mls of serum-free SLM. The medium was harvested after these 72 hours and the required protein purified, analysed and bioassayed as required. In order to obtain valid controls when assaying the above proteins, mock COS 7 cell transient transfections were also performed either without a plasmid or with the parent pMTx vector.

2.12.2. Stable transformations.

Stably transformed CHO cell lines expressing human MIP-1α were obtained from Genetics Institute, Cambridge, Mass., USA, These methotrexate resistant cells were cultured in the presence of methotrexate unless the collection of MIP-1α containing supernatant was required, when the cells were grown in serum-free medium in the absence of methotrexate.

2.13. MIP-1α protein purification and preparation for bioassays .

The cell supernatants were collected, and then centifuged at 2000 rpm to remove any cellular debris. The supernatants were then filtered through 0.2μm filters and stored at -20°C until required for the purification of MIP-1α. The purification of MIP-1α from cell supernatants has been previously described (Graham et al 1992).

The purification procedure used in these analyses involved a combination of heparin-sepharose and blue-sepharose affinity chromatography. The supernatant was initially loaded on to a heparin sepharose affinity column (Pharmacia Ltd) to which the MIP-1α protein bound. The column was then washed with 0.1M NaCl/0.02M Tris pH7.6 to elute the heparin non-binding proteins (fraction H1), and then the MIP-1α and other heparin-binding proteins were eluted with 0.5M NaCl/0.02MTris pH7.6 (faction H2). This H2 fraction contained partially purified MIP-1α. This eluted material in the H2 fraction, when further purification was required, was loaded directly onto a blue-sepharose affinity column (Pharmacia Ltd) to which the MIP-1α bound avidly. The column was then washed with 2M NaCl/ 0.02M Tris pH 7.6 to remove non-binding proteins (fraction B1) and the material was eluted using 4.5M $MgCl_2$ (fraction B2). This B2 fraction comprised MIP-1α purified to a single band on a silver stained SDS-PAGE gel. Where H2 or B2 were to be used in keratinocyte or CFU-A bioassays they were first desalted using a sephadex G25 column into phosphate buffered saline. When the material was to be used for electospray mass spectroscopy B2 was desalted into HPLC grade water. All the purification and desalting procedures were performed on the Pharmacia LCC-500 FPLC system.

The H2 fraction was also purified further using reverse phase chromatography on the FPLC system. A 1 ml Resource (Pharmacia) reverse phase column was used. The system was run at 1 ml/min and the proteins were eluted off the column using a gradient of 0 to 100% acetonitrile and a gradient volume of at least 15 column volumes (15mls). The solvent

system used was 0.1% TFA in degassed double distilled water (Solvent A) and 0.1% TFA in degassed acetonitrile (Solvent B). After loading the sample onto the column, the column was first washed with 5 column volumes (5mls) of solvent A and then the 0-100% gradient of acetonitrile/ 0.1%TFA applied. All the eluted fractions were collected and stored at -20°C. The fractions were then analysed by Western blotting and silver staining. If the fractions were to be bioassayed, the samples were dried down under vacuum, redissolved in PBS and then applied to the cells of interest.

2.14. Protein characterisation.

2.14.1. SDS Polyacrylamide gel electrophoresis.

Polyacrylamide gels were prepared using a commercially available acrylamide/ bisacrylamide stock solution: 30% w/v acrylamide / 0.8% w/v bisacrylamide (Severn Biotech Ltd.) as described in "Molecular Cloning: A Laboratory Manual" (Sambrook, Fritsch and Maniatis). 15-17.5% SDS-PAGE gels were usually used. The appropriate volumes of acrylamide/bisacrylamide mix, water, 1.5M Tris pH 8.8, 10% SDS, 10% ammonium persulphate and TEMED (Sigma) were mixed and poured between two minigel glass plates. Approximately 1ml of water saturated iso-butanol was layered on top of the solution to enable a smooth interface between stacking and resolving gels to be formed, and the gel was left to set at room temperature. A 5% stacking gel was prepared essentially as above but with differing amounts of additives and using a 1M Tris buffer pH 6.8 (again as described in Maniatis). The water saturated butanol was poured off, and the top of the gel thoroughly washed using distilled water. Approximately 3-4mls of stacking gel solution was then poured between the glass plates overlaying the solid gel and an appropriate comb was inserted to construct wells. This was then left to set for at least one hour. When the wells had set the comb was carefully removed. Samples were prepared for electrophoresis by adding 20μl of each sample to 5μl SDS/DTT and 5μl of gel loading buffer (50% bromophenol blue solution/ 50% glycerol) in 1.5ml Eppendorf tubes and heated at 100^{o}C for 3 minutes to denature and reduce the proteins. Samples were loaded onto the gel along with molecular weight rainbow markers (Amersham) to enable sizing of discrete bands and to check transfer had occurred during Western blotting.

Glycine SDS-PAGE gel electrophoresis was carried out at room temperature in SDS-PAGE electrophoresis buffer (28.8g glycine, 6.05g tris.HCl, 2g of SDS and made up to 2 litres with distilled H2O) at 100-150V until the blue dye front almost reached the bottom of the gel.

Tricine SDS-PAGE gels were performed when higher resolution in the range 5 to 20kDa was required (Schagger and Von Jagow 1987). In these tricine gels a spacer gel section was set in between the separating and stacking gels. The composition of the separating, spacer and stacking gels are outlined below.

			|----------Separating Gels----------|		
	Stacking Gel 4%T, 3%C	Spacer gel 10%T 3%C	10%t 3%C	16%T 3%C	16.5%T 6%C	16.5%T,6%C with 6M urea
49.5%T, 3%C solution	1 ml	6.1ml	6.1ml	10ml	-	-
49.5%T, 6%C solution	-	-	-	-	10ml	10ml
Gel buffer	3.1ml	10ml	10ml	10ml	10ml	10ml
Glycerol	-	-	4g	4g	4g	-
Urea	-	-	-	-	-	10.8g
Add water to a final volume of	12.5ml	30ml	30ml	30ml	30ml	30ml

These tricine discontinous SDS PAGE gels were made up as outlined for the glycine SDS-Page gels above. The buffers used in this system are outlined below.

Buffer	Tris (M)	Tricine (M)	pH	SDS(%)
Anode buffer	0.2	-	8.9	-
Cathode buffer	0.1	0.1	8.25	0.1
Gel buffer	3.0	-	8.45	0.3

The gel was run at 100V continously until the dye-front had reached the bottom of the gel. The gel was then removed from the gel-tank and either silver stained or blotted onto nitrocellulose paper for Western blotting.

2.14.2. Silver staining.

The protein bands were fixed by placing the polyacrylamide gel in a solution containing 50% methanol and 10% acetic acid for ten minutes. This solution was then discarded and the gel placed in 10% ethanol/10% acetic acid for another 10 minutes. Again this solution was discarded and the gel was placed in 10% gluteraldehyde for 10 minutes. Following this the gel was washed at least six times in distilled water over approximately ten minutes.

The gel was then placed in a solution of 40μM DTT for ten minutes (A 1M DTT stock solution was stored at -20°C). The solution was again discarded and the gel incubated in 0.1% silver nitrate for a further ten minutes. The gel was then rinsed several times with distilled water and then placed in a small amount of developer solution until this became discoloured. (The developing solution was made up by dissolving 15g anhydrous sodium carbonate in 500mls of water, and then adding 500μls of formaldehyde.) The developer solution was replaced with fresh solution and the gel developed until the protein bands appeared. The gel was then washed in water to prevent further developing. Silver stained gels were used to assess the purity and the protein content of all conditioned media.

2.14.3. Western blotting.

The protein (usually MIP-1α) content of cell conditioned media was also analysed by Western blotting. The contents of the gel were transferred to nitrocellulose membranes (Schleicher & Schuell) by semi-dry blotting using minigel electroblotting equipment (Biotech Instruments Ltd.) at a constant current of 8mA/cm^2 gel area for 30 minutes as follows. Briefly, many pieces of Whatman 3MM blotting paper were cut to match the dimensions of the polyacrylamide gel (usually 6cm x 9cm), as was a piece of nitrocellulose. Six pieces of blotting paper were soaked in electroblotting buffer and layered on top of each other. Any air bubbles were removed by rolling using a plastic pipette. The nitrocellulose membrane was then placed on the filter paper followed by the polyacrylamide gel, again removing any air bubbles. Six pieces of blotting paper soaked in the same electroblotting solution were placed on top of the gel and the apparatus assembled. The electroblotter was then turned on and run at the above mentioned conditions. After blotting, the membrane was incubated in BLOTTO solution (5% Marvel dried milk in PBS, 0.1% NP40) for 30 minutes to block non-

specific binding sites. The membrane was then exposed to the primary polyclonal goat anti-MIP-1α antibody at the appropriate dilution (usually 1:1000) in 10mls of BLOTTO for 1 hour. This was discarded and then membrane washed in several changes of BLOTTO for 45 minutes. The membrane was then incubated with the secondary anti-goat IgG antibody conjugated to horse radish peroxidase in 10mls of BLOTTO for 1 hour after which this was discarded and the membrane washed in several changes of PBS/ 0.1% Tween for 30 minutes. Antibody binding was then visualised using an enhanced chemiluminescence (ECL) kit (Amersham), which involved agitating the membrane in ECL solutions for 60 seconds. The membrane was then wrapped in Saranwrap and exposed to X-OMAT AR X-ray film for the appropriate time to enable visualisation of protein bands.

2.14.4. TGF-β1 protein quantitation using ELISA

TGF-β1 protein content of partially purified MIP-1α derived from the transient transfection of COS 7 cells using DEAE-Dextran was determined using a commercially available "sandwich technique" ELISA kit (R&D systems, UK). This kit adopts an alternative protocol to traditional ELISA based methodology in that initial TGF-β binding to the plate is accomplished via TGF-β type II receptor adherent to the well (rather than an antibody) which recognises all TGF-β isoforms. The kit then specifically detects TGF-β1 via a polyclonal secondary antibody conjugated to horse radish peroxidase. As only active TGF-β is capable of binding to the type II receptor, this method necessitates acid activation of TGF-β present in conditioned media samples. 1ml of transfection supernatant was acid treated by adding 200μl of 1N HCl for 10 minutes and then neutralised by adding 200μl of 1.2N NaOH/0.5M HEPES. As a control, a standard curve of pure TGF-β1 was prepared by diluting 500μl of a 2000pg/ml stock into 500μl of RD5I diluent (see manufacturers instructions). Serial dilutions from 1000pg/ml to 31.25pg/ml were then carried out. Although human TGF-β1 was used as a control, the assay kit detects murine or primate TGF-β1 with equivalent accuracy. 200μl of standards and samples were then loaded in duplicate into a 96 well plate, covered with a plastic film and incubated at room temperature for 3 hours. Plate contents were discarded and each well washed x 3 with 400μl of wash buffer. 200μl of anti-TGFβ1 conjugate was then added to each well and incubated at room temperature for a further 90 minutes. Each

well was then washed x 3 with 400μl of wash buffer after which 200μl of substrate solution was added to each well. The plate was incubated at room temperature for 20 minutes to allow colour development and 50μl of stop solution was then added to each well. The optical density of each well was then determined using a Beckman DU 650 spectrophotometer set to 450nm. Readings at 570nm were also taken and subtracted from the 450nm readings to correct for optical imperfections in the polystyrene microtiter plate.

2.15. ^{125}I labelling of MIP-1α protein

MIP-1α protein preparations were labelled using Iodo-gen (Pierce, U.S.A.), a commercially available iodination reagent which resembles a four-fold chloramine T molecule. 10μg of MIP-1α in PBS was incubated with 10μg of immobilised iodogen and 1mCi Na^{125}I (New England Nuclear) in Eppendorf tubes for 15 minutes on ice. All work involving ^{125}I was carried out behind a protective lead impregnated screen. Unincorporated iodine was then separated from labelled protein by applying the reaction mixture to a disposable desalt column (GF5 excellulose columns; Pierce) and eluting with PBS. 500μl fractions were collected and counts per minute assessed in a gamma counter to detect the peak of protein associated radioactivity. The three most active fractions were then pooled yielding a solution of MIP-1α protein labelled to high specific activity ($2.5x10^7$ cpm/mg). The integrity of the labelled protein was then checked by SDS-PAGE. Iodination does not affect the biological potency of the protein in the CFU-A assay (Graham *et al* 1993). The radiolabelled preparations were used to analyse the purity of the partially purified MIP-1α (Results section).

2.16. Electrospray Mass spectroscopy.

Electrospray mass spectroscopy was used to analyse the molecular weight of both bacterial and COS 7 cell derived MIP-1α . Electrospray mass spectroscopy was carried out with the help of Tino Krell at the Department of Biochemistry, University of Glasgow. Mass spectroscopy was performed on a VG platform quadrupole mass spectrometer (2-3000 amu range) fitted with a pneumatically assisted electrospray (ionspray source) and controlled via the MG mass-Lynx software (VG Biotech Ltd, Altrincham, Cheshire, UK). Carrier solvent (1:1 (v/v) acetonitrile/water) infusion was controlled at 10 ml/min using a Harvard syringe pump

(Harvard apparatus, Ma.,USA.). Protein samples were dissolved in carrier solvent containing 2% formic acid, 0.1% acetic acid (to minimize MIP-1α aggregation) and at a concentration of 20 pmol/μl, centrifuged at 5000x g for 2 minutes and then 10-20 μl samples injected into the carrier stream. Capillary voltages were between 2.8 and 3.2kV, extraction cone voltages 20-30V, and the focusing cone voltage offset by +10V. The source temperature was set at 65°C, the nebulising gas flow at 10l/h, and the drying gas flow at 250l/hr. Lens stack voltages were adjusted to give the maximum ion currents. The m/z range 700-1500, which contained >95% of the signal intensity for protein samples, was scanned at least 10 times with a sweep time of 5 seconds. The instrument was calibrated over this Mr range immediately before use with horse heart myoglobin.

Results.

3.1. Introduction.

MIP-1α is known to inhibit the proliferation of specific populations of haemopoietic stem cells. A partially purified form of MIP-1α, derived from the transient transfection of COS 7 cells, is also able to reversibly inhibit the proliferation of clonogenic cultured epidermal keratinocytes (Parkinson et al 1993). This partially purified preparation of MIP-1α (90-95% pure) which will be called GI MIP-1α was generated by colleagues at the Genetics Institute (Cambridge, Mass.,USA) and partially purified by Dr GJ Graham. It was used because when these experiments were first performed a bacterial recombinant form of MIP-1α was not available. When a bacterial recombinant form of MIP-1α (bMIP-1α) became commercially available this was found to be unable to inhibit the proliferation of keratinocytes in the KGM assay although in common with GI MIP-1α it did inhibit the proliferation of cells in the haemopoietic CFU-A and CFU-S assays. Surprisingly however a specific polyclonal antibody to this bacterial recombinant chemokine was shown to be able to fully reverse the keratinocyte inhibitory activity of GI MIP-1α implicating this chemokine in the inhibition of keratinocyte proliferation. However the inactivity of bMIP-1α on the KGM assay suggested that this chemokine requires some form of activation to inhibit keratinocyte proliferation.

Since I only had access to a very limited supply of GI MIP-1α, the first step in investigating the keratinocyte inhibitory activity of this chemokine involved the generation of this protein by the transient transfection of COS 7 cells in our laboratory (Lab MIP-1α) .

3.1.1. The Preparation of Lab MIP-1α.

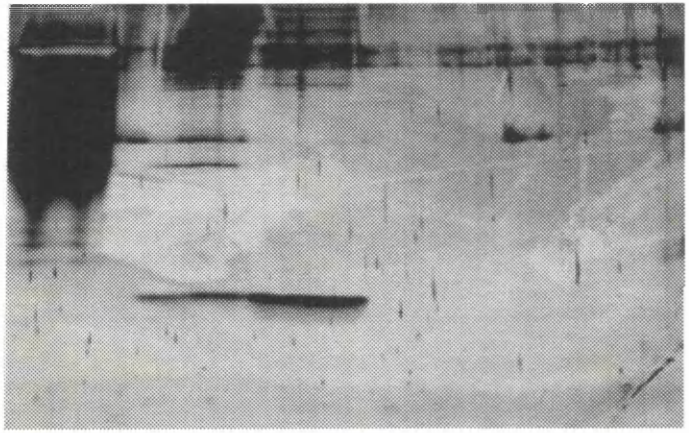
COS 7 cells were transfected with the plasmid pMTx.MIP-1α (murine MIP-1α cDNA) as described in Section 2.14.1. The supernatant obtained from this DEAE dextran transient transfection was then partially purified by heparin sepharose affinity chromatography (Graham et al 1992). The murine MIP-1α containing fraction (H2) was then desalted into PBS and analysed by SDS-PAGE, silver staining and Western blotting *(figure 1a).*

Figure 1a:

A Silver Stained SDS-PAGE 17.5% Gel of Lab MIP-1α.

H1: Heparin non-binding fraction.
H2 (9,10) : Heparin binding fractions eluted sequentially off heparin column.

8KDa MIP-1α band.



H1 H2(9) H2(10) .

H1 represents the non-heparin binding protein fraction and H2 fractions 9 and 10 the heparin binding fractions. These two H2 fractions were pooled, and termed Lab MIP-1α. Lab MIP-1α was slightly less pure than GI MIP-1α as can be seen in *figure 1b*. However this Lab MIP-1α preparation is still relatively pure as many of the higher molecular weight bands seen in the silver stained gel in figure 1b are gel artefacts since they are also present in the pure bacterial MIP-1α preparation. Lab MIP-1α was analysed by Western blotting and the approximate concentration of MIP-1α in these partially purified preparations was determined. Furthermore the biological activity of Lab MIP-1α was tested in the haemopoietic CFU-A assay in order to further quantitate the preparation.

3.1.2. The activity of Lab MIP-1α on CFU-A colony formation.

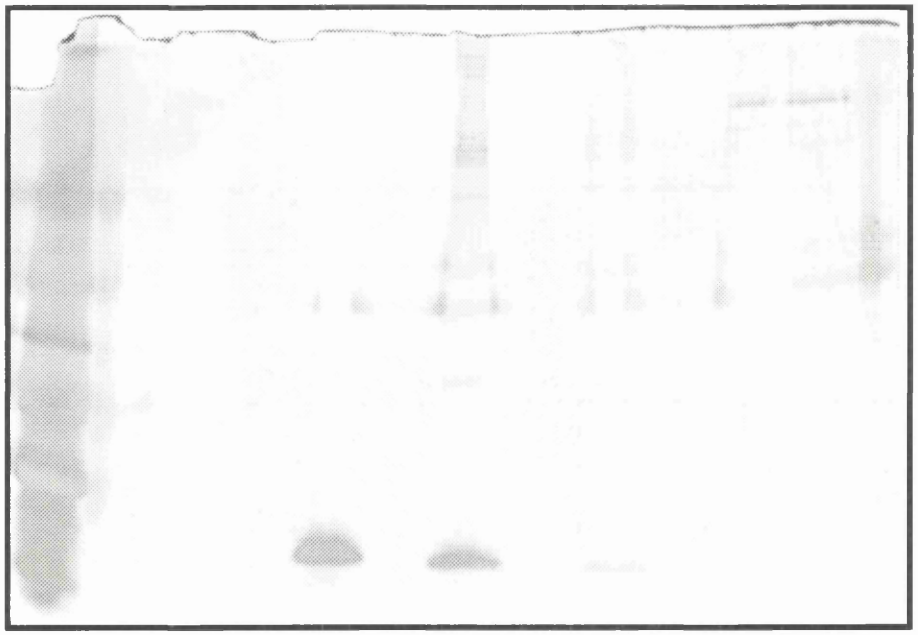
Lab MIP-1α and bacterial MIP-1α were analysed in the haemopoietic CFU-A assay. The results which are presented in figure 1c show that the the bacterial recombinant protein was able to inhibit the CFU-A assay with an ED 50 of approximately 10ng/ml. Lab MIP-1α was also able to inhibit colony formation in the CFU-A assay with an ED50 of 10-20μls of Lab MIP-1α *(figure 1c)*. These results are in agreement with previous results (Graham et al 1990).

3.1.3. Effects of Lab MIP-1α on keratinocyte colony formation.

Lab MIP-1α was then tested in the KGM assay to confirm the kerato-inhibitory activity of this protein. The assay was performed using a range of concentrations of both Lab and GI MIP-1α. The results are shown in *figure 2* and show that both MIP-1α preparations are able to inhibit keratinocyte colony formation with similar potencies. Half maximal inhibition was obtained with a MIP-1α concentration of approximately 30ng/ml. *Figure 3* demonstrates the keratinocyte colonies in the KGM assay: both control colonies and inhibited keratinocytes are displayed. bMIP-1α (R&D systems) was also analysed in these assays *(figure 2).* This preparation of murine MIP-1α is inactive in the KGM assay. bMIP-1α was unable to inhibit keratinocyte proliferation even at concentrations as high as 10μg/ml.

FIGURE 1b

Silver stained 15% SDS-PAGE gel of GI H2 and LAB H2 MIP-1α.



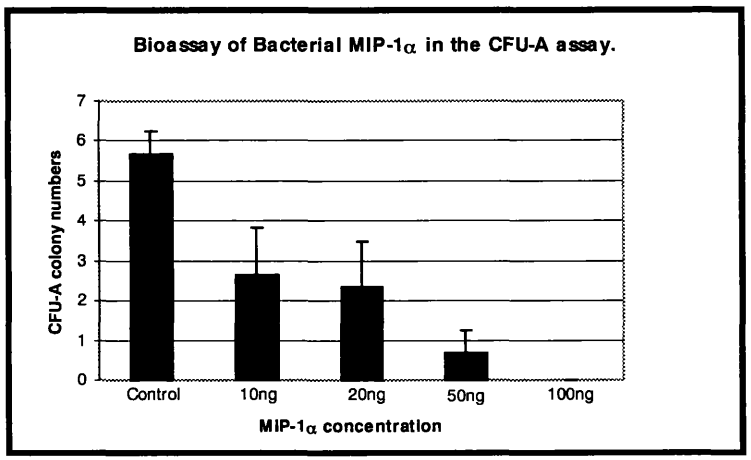
- 8KiloDaltons.

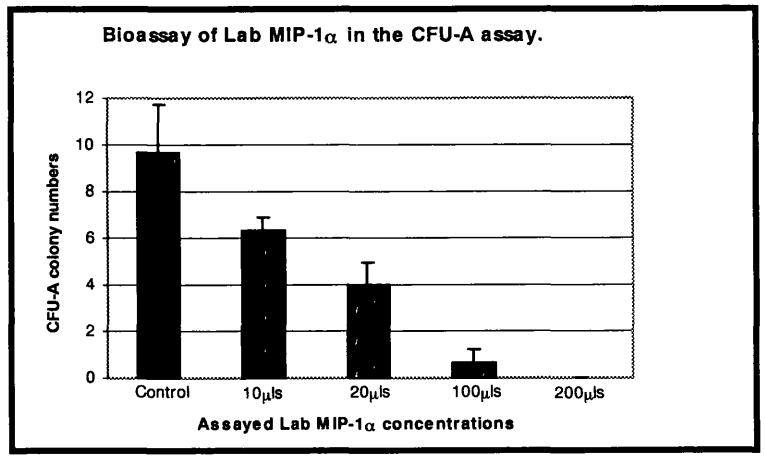
MWM GIH2 LAB H2 B*

* B is bacterial recombinant MIP-1α.

Figure 1c.

Both Bacterial and LAB MIP-1α inhibit the CFU-A assay.

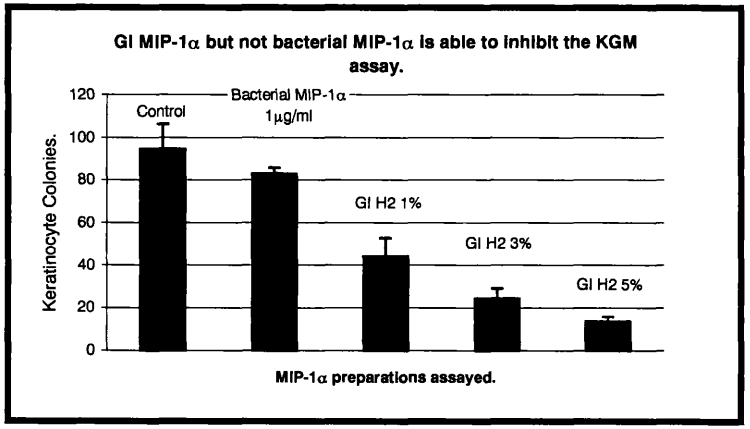


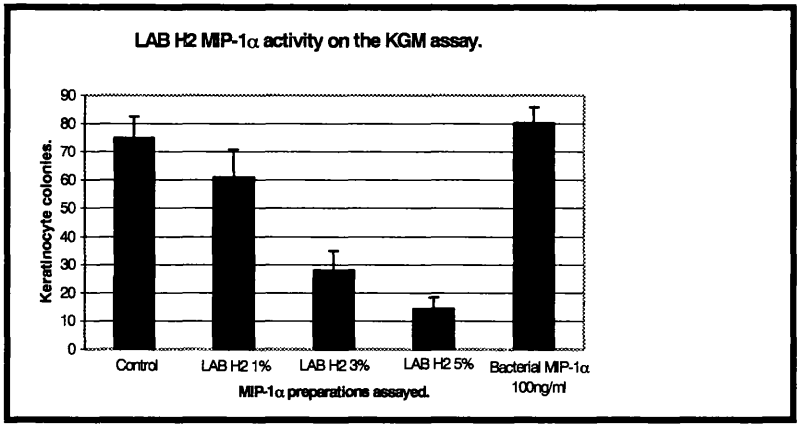


These results represent three different experiments. CFU-A colonies greater than 2mm were counted. The above results represent the number of colonies cultured on a 35mm Petri dish from 1x 10^4 total bone marrow cells.

Figure 2

LAB H2 and GI H2 both inhibit clonogenic keratinocyte proliferation.



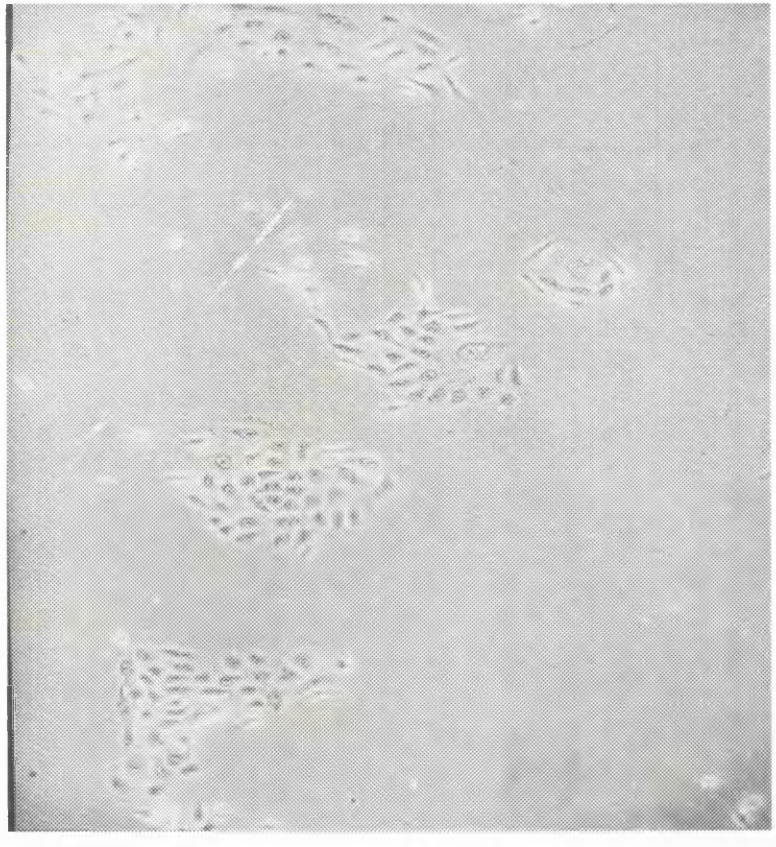


- Both GI H2 and LAB H2 were desalted into phosphate buffered saline and diluted into KGM. Both preparations inhibited the clonogenic keratinocyte assays at approximately equivalent concentrations as determined by SDS-PAGE analysis.
- A wide range of bacterial murine MIP-1α concentrations was tested. bMIP-1α did not significantly inhibit the KGM assay at concentrations ranging from 1ng/ml to 10μg/ml.
- These results are representative of three separate assays.

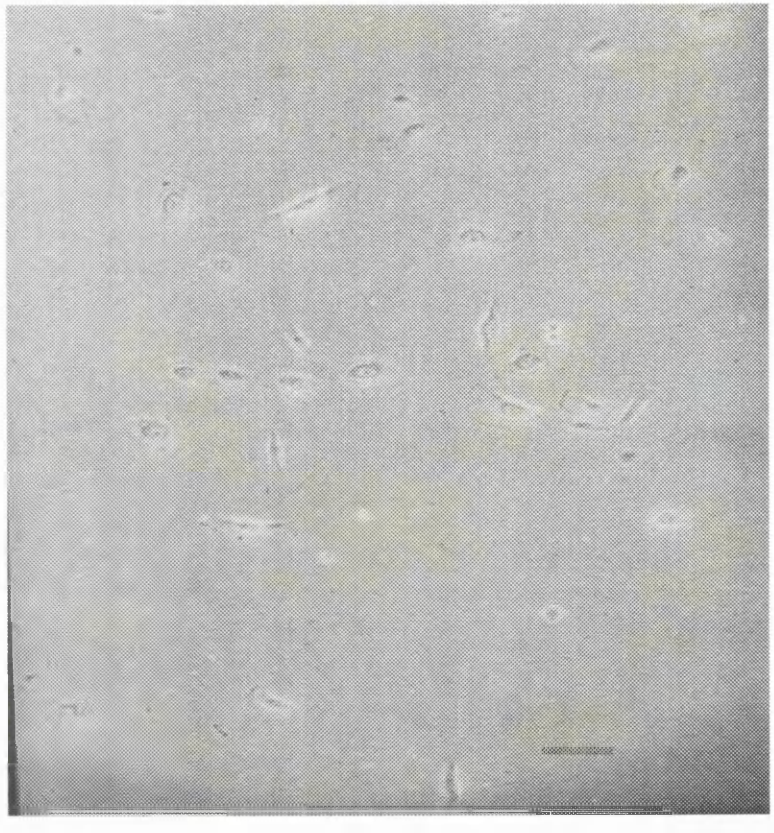
Figure 3.

Human epidermal keratinocyte colonies in KGM assay.

Uninhibited Colonies.



Inhibited keratinocytes.



3.1.4. The inhibitory activity of LAB MIP-1α is reversed by a specific polyclonal antibody to bacterial murine MIP-1α.

The activity of GI MIP-1α on keratinocyte proliferation can be reversed by a specific polyclonal antibody to bacterial murine MIP-1α (Parkinson et al 1993). The kerato-inhibitory activity of LAB MIP-1α can also be reversed by this specific polyclonal anti-murine MIP-1α antibody (obtained from R&D systems) as shown in figure 4. Although this reversal is not complete it is statistically significant ($p < 0.01$). It is therefore clear that as seen with GI MIP-1α, the inhibitory activity of Lab MIP-1α is primarily due to the MIP-1α protein.

3.1.5. Summary.

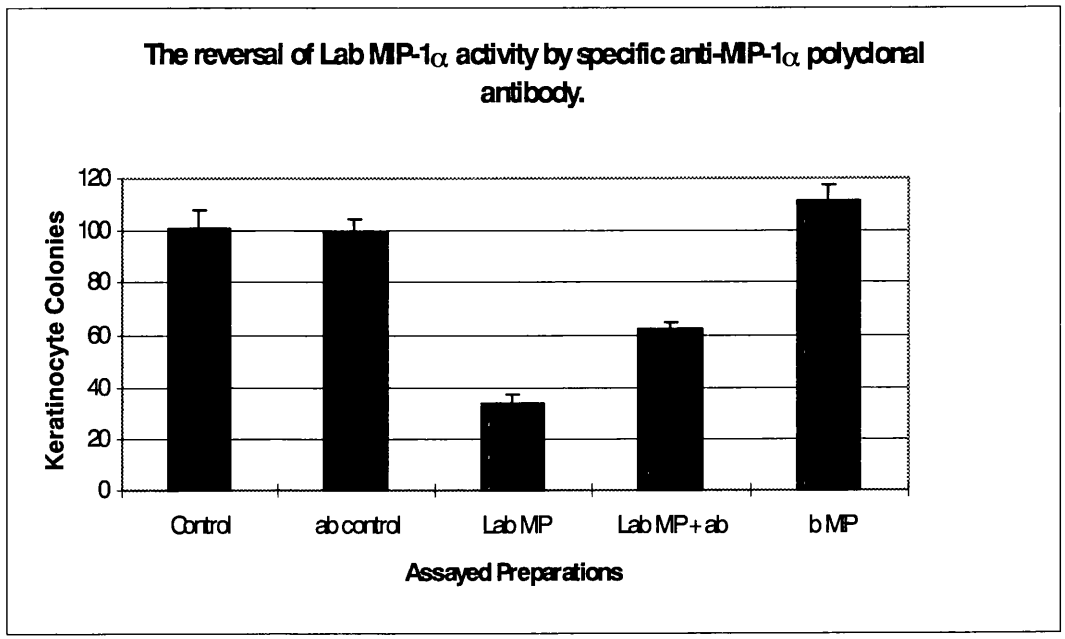
These experiments were carried out to ensure that MIP-1α derived by the dextran transient transfection of COS 7 cells in our laboratory (Lab MIP-1α) was able to inhibit clonogenic keratinocyte proliferation in the KGM assay, since only a limited amount of GI MIP-1α material was available. A keratinocyte inhibitory form of MIP-1α, Lab MIP-1α, functionally identical to GI MIP-1α was now readily available in the laboratory. Therefore GI MIP-1α and Lab MIP-1α were used interchangeably in the experiments now described (these two preparations will from now on be termed COS MIP-1α), which attempt to elucidate why bacterial MIP-1α is inactive in the KGM assay, while COS MIP-1α is a potent inhibitor of clonogenic keratinocyte proliferation.

Although the reason for this discrepancy was not clear, since the COS MIP-1α preparations were impure, it was possible that MIP-1α required an accessory factor to inhibit keratinocyte proliferation. On the other hand it was possible that the mammalian cell expression system was able to activate MIP-1α through some form of structural modification which the bacterial expression system was unable to effect. Furthermore these results also suggested that the keratinocyte and the haemopoietic stem cell (CFU-A) have different MIP-1α receptors.

Figure 4.

Anti-MIP-1α antibody is able to reverse the activity of LAB MIP-1α.

- 1mg of antibody to bacterial mu MIP-1α (R&D) was diluted in 1ml of PBS. 100μls of antibody was then incubated with the COS 7 cell derived LAB MIP-1α at 37°C for 1 hour prior to addition to the assay.



The above antibody reversal is statistically significant: p=0.0082 (Paired t-test)

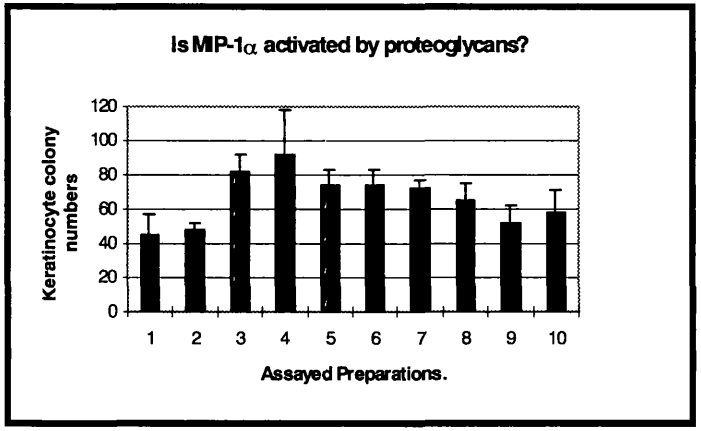
3.2. Putative accessory factors.

3.2.1. Proteoglycans.

A number of studies have shown that MIP-1α, like all the other members of the chemokine family (Witt and Lander 1994), is able to bind a number of proteoglycans, including heparan-sulphate as well as heparin. Furthermore it has been previously suggested that proteoglycans may be able to modulate the function of a number of cytokines (Ruoslahti and Yamaguchi 1991). Since previous studies with the CXC chemokine IL-8 have shown that heparan sulphate is able to modulate the activity of this chemotactic cytokine (Webb et al 1993), we postulated that the interaction between this proteoglycan and the chemokine may activate the COS preparation's keratoinhibitory activity. To investigate this hypothesis, bMIP-1α was assayed (100ng/ml) in the presence of different concentrations of heparin, heparan sulphate and chondroitin sulphate A and B. Only the results obtained with the highest proteoglycan dose used are presented (1μg/ml), however similar results were obtained at all the concentrations tested (10ng/ml, 100ng/ml, 1μg/ml). These results, presented in *figure 5,* show that these proteoglycans do not function as accessory factors for bacterial MIP-1α in the KGM assay at the concentrations tested . These results do however suggest that these proteoglycans alone can alter the colony forming efficiency of this assay. In fact heparin sulphate significantly increased keratinocyte colony formation (p=0.006). None of the other proteoglycans tested altered keratinocyte proliferation significantly). The mechanism of the increased colony-forming efficiency seen in the described experiments was not clear. Interestingly however previous studies have suggested that in the absence of EGF, heparin sulphate can inhibit the cells in the KGM assay (IC50= 500ng/ml) although this inhibition is completely overridden by EGF(Cook et al 1991). However other experiments have also shown that the mitogenic activity of EGF is not modulated by heparin (Aviezer et al 1994). It is possible however that heparan sulphate is either able to modify the activity of other mitogenic and/or inhibitory factors in the KGM assay or that it is able to decrease cell loss by functioning as a cell survival factor. Overall however it is clear that the bioactivity of bacterial MIP-1α in this assay is unaltered by the proteoglycans tested.

Figure 5.
Bacterial MIP-1α is not activated by proteoglycans.

•	Column 1	Control
•	Column 2	Bacterial murine MIP-1α (b)
•	Column 3	Heparin
•	Column 4.	Heparin and b
•	Column 5.	Heparan sulphate
•	Column 6.	Heparan sulphate and b
•	Column 7.	Chondroitin sulphate A
•	Column 8.	Chondroitin sulphate A and b
•	Column 9.	Chondroitin sulphate B.
•	Column 10.	Chondroitin sulphate B and b



The bacterial MIP-1α was assayed at a concentration of 100ng/ml.The proteoglycans were assayed at a number of different concentrations ranging from 10ng/ml to 1 μg/ml. Only the results obtained with the higher concentrations of proteoglycans are presented. However similar results were obtained at all the concentrations tested.
The increased cloning forming efficiency seen with heparin sulphate was statistically significant (p= 0.006),

3.2.2. **Do COS 7 cells secrete a co-factor for the HEK inhibitory activity of MIP-1α?**

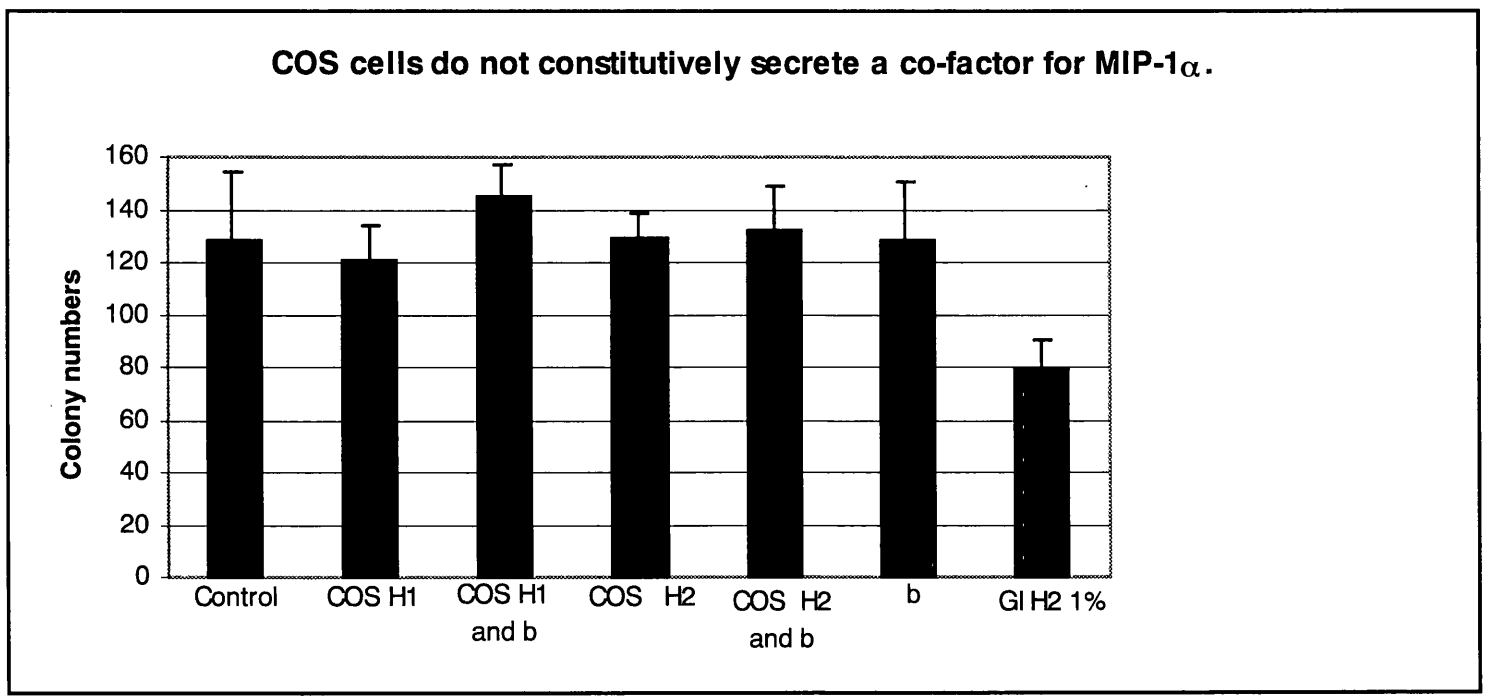
If MIP-1α does require an accessory factor to inhibit keratinocyte proliferation, the above results suggest that COS 7 cells secrete this putative co-factor. In order to investigate this further the proteins secreted by untransfected COS 7 cells were analysed to determine whether they could function as co-factors for bMIP-1α in the KGM assay.

a) Constitutively expressed heparin-binding accessory factor?

Since the transient transfection of COS 7 cells by DEAE Dextran involves the collection of expressed MIP-1α in serum-free medium over a 72 hour period, 10 mls of COS 7 cell serum-free conditioned medium was collected after 72 hours of culture. This medium was then partially purified by heparin sepharose affinity chromatography and both the heparin binding (COS H2) and the heparin non-binding protein (COS H1) fractions were desalted into phosphate buffered saline. Titrated quantities of these protein preparations COS H1 and COS H2 (1%, 3%, 5%) were then assayed in the presence or absence of bMIP-1α (assayed concentration 100ng/ml). The controls for this experiment included keratinocytes cultured in the presence of either bacterial recombinant MIP-1α alone (100ng/ml), or keratinocytes cultured with the COS H1 and H2 fractions alone (1%, 3%, 5%). The results of these experiments are presented below in *figure 6.* They suggest that COS 7 cells do not constitutively secrete a co-factor for this chemokine at the concentrations tested.

Figure 6.

COS 7 cells do not constitutively secrete an accessory factor for MIP-1α.



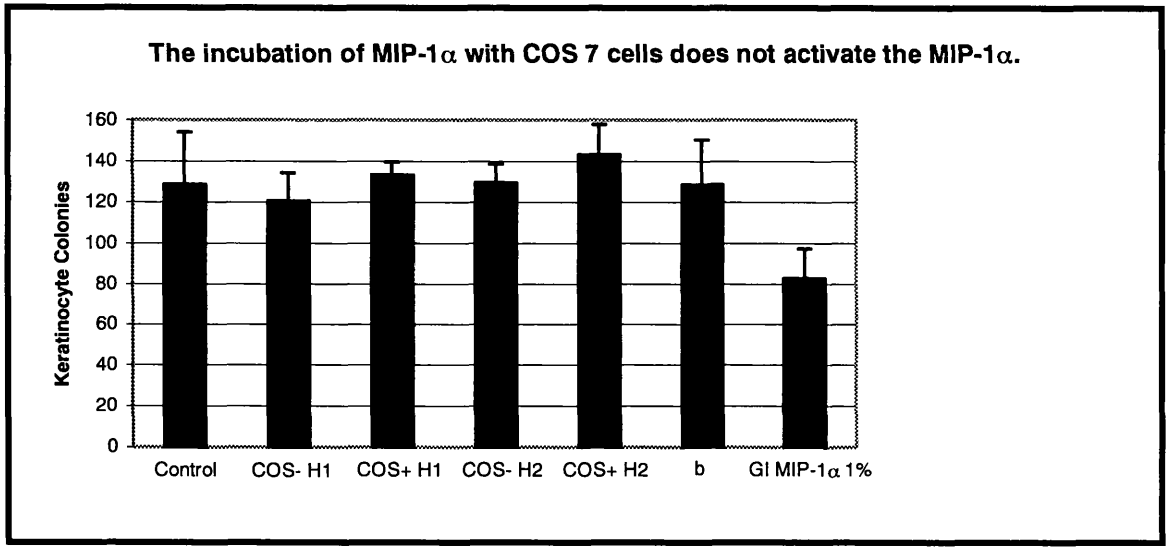
These results represent three different experiments. The COS 7 cell proteins were assayed at 1%, 3% and 5% dilutions, since GI and Lab MIP-1α were active at these dilutions. The COS 7 cell derived proteins had no effect at any of the concentrations tested. Only the results obtained with the highest concentration tested (5%) are presented. The bacterial murine MIP-1α (b) was assayed at a concentration of 100ng/ml.

b) Does MIP-1α stimulate COS 7 cells to secrete a MIP-1α co-factor?

Although COS 7 cells do not appear to constitutively secrete a co-factor, it is possible that these cells will only produce this putative molecule in the presence of MIP-1α. To investigate this a range of concentrations of bacterial recombinant murine MIP-1α (100ng/ml, 1μg/ml, 10μg/ml) were incubated for 72 hours in the presence of sub-confluent COS 7 cells cultured in 10 mls of serum-free medium. This supernatant containing MIP-1α ('COS+') was then collected, partially purified by heparin affinity chromatography, desalted and bioassayed on the keratinocyte assay at three different dilutions (1%, 3%, 5%). An identical flask of sub-confluent COS cells was at the same time cultured in the absence of bacterial recombinant MIP-1α in 10 mls of serum free medium and this 'COS- ' supernatant was also partially heparin purified, desalted and bioassayed as a control to the 'COS+' preparation at identical dilutions. Only the results obtained at the highest concentrations assayed (5%) are presented in *figure 7* since at the three concentrations tested, the incubation of bMIP-1α with COS 7 cells did not appear to activate the chemokine. These results suggested that in serum-free medium COS 7 cells do not secrete a co-factor for MIP-1α. Furthermore if MIP-1α is being activated by some form of structural activation, these results suggest that the incubation of bMIP-1α with COS 7 cells in serum-free medium does not result in this functional activation.

Figure 7.

The incubation of MIP-1α with COS 7 cells does not generate kerato-inhibitory activity.



Control	5% Phosphate buffered saline (PBS)
COS- H1	5% of non-heparin binding COS proteins in PBS
COS+H1	5% of non-heparin binding proteins in PBS after 72h incubation of bacterial mu MIP-1α on COS 7 cells.
COS- H2	5% of heparin-binding COS proteins in PBS.
COS+H2	5% of heparin-binding proteins in PBS after 72hr incubation of bacterial mu MIP-1α on COS 7 cells.
b	5% of bacterial murine MIP-1α at 1μg/ml in PBS (50ng/ml).
GI MIP-1α	Assayed at 1% (control).

c) Do COS 7 cells only secrete a co-factor for MIP-1α during transient transfection?

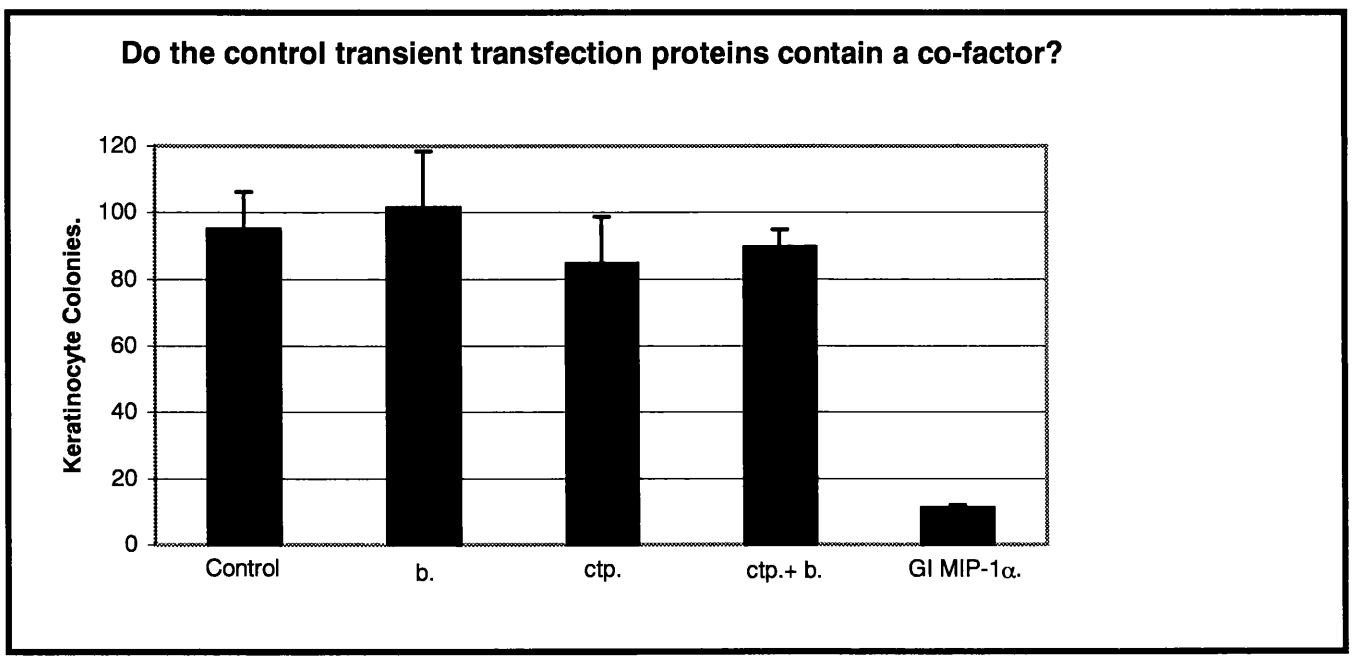
The above results suggest that COS 7 cells do not secrete an accessory factor for MIP-1α. The dextran transient transfection may however stimulate COS 7 cells to secrete this putative co-factor. To investigate this a mock dextran transient transfection was performed using the parental plasmid vector (pMTx). The control dextran transfection proteins in the culture media obtained were partially purified by heparin affinity chromatography and desalted into PBS. These proteins were assayed in the presence or absence of bacterial recombinant MIP-1α in the KGM assay. The results are presented in *figure 8* and show that at the concentrations tested the control dextran transient transfection partially purified proteins have no detectable effect on the activity of bMIP-1α in the inhibition of clonogenic keratinocyte proliferation. Therefore it appears that that COS 7 cells do not secrete an accessory factor for MIP-1α during the mock dextran transient transfection.

d) Does the inactive bacterial MIP-1α alter the bioactivity of COS 7 cell MIP-1α?

The above results all appear to suggest that COS 7 cells do not secrete a MIP-1α co-factor. To test this co-factor hypothesis further, it was decided to investigate the effect of the inactive bacterial protein on the active COS 7 cell MIP-1α preparation. If an accessory factor is present in functional excess of the MIP-1α protein in this active preparation, a sub-inhibitory dose of the COS 7 MIP-1α preparation may be made more potent by the addition of the recombinant bacterial MIP-1α. Sub-inhibitory concentrations of COS 7 cell MIP-1α were therefore bioassayed in the presence or absence of a titration of inactive bacterial murine recombinant MIP-1α (10μg/ml; 1%, 3% and 5% dilutions). The controls used were the identical sub-inhibitory concentration of COS 7 cell MIP-1α alone, or bacterial MIP-1α alone, while the positive control used was the COS 7 cell derived bioactive MIP-1α at a ten-fold higher concentration. The results of these experiments are presented in *figure 9* below.

Figure 8

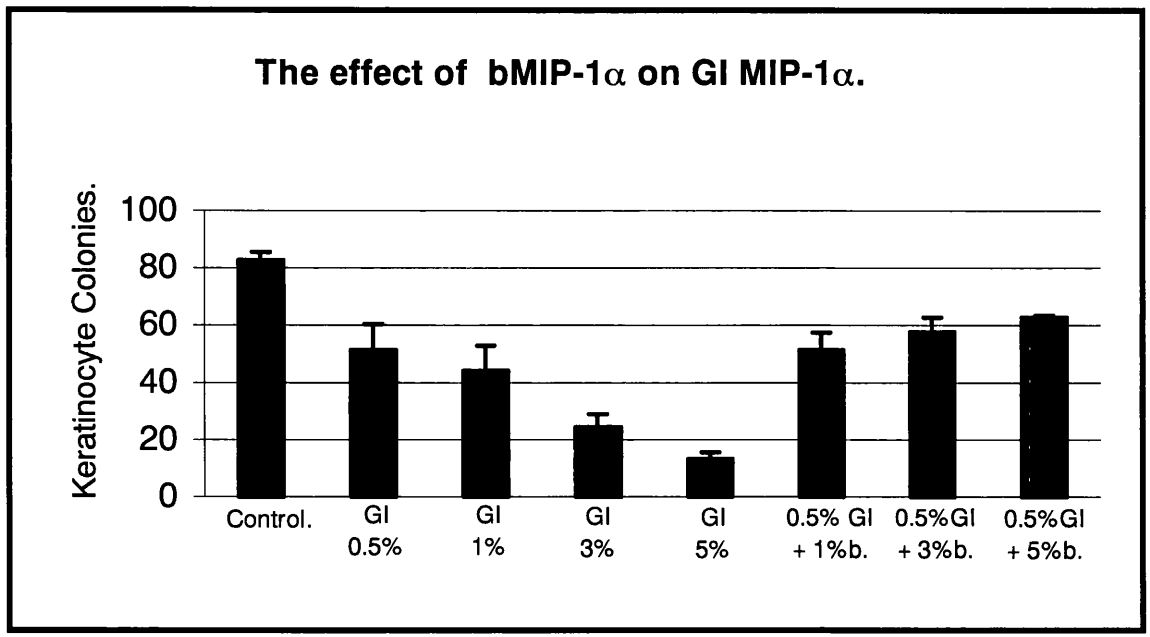
COS 7 cells do not secrete an accessory factor for MIP-1α during transient transfection.



Control	**300μls of PBS.**
b.	**bMIP-1α 100ng/ml.**
ctp.	**Control dextran transfection heparin purified proteins (5% dilution).**
ctp. + b.	**ctp. and bMIP-1α at 100ng/ml.**
GI **MIP-1α**	**5% dilution**

Figure 9.

The addition of bacterial MIP-1α to low concentrations of GI MIP-1α does not significantly alter the activity of the active preparation.



GI MIP-1α (GI) was assayed in the presence or absence of bacterial recombinant MIP-1α (b) at doses varying from quasi sub-inhibitory doses (0.5%) to the fully inhibitory dose (5%). Bacterial MIP-1α (10μg/ml) was added at 1, 3 and 5% dilutions. Although these results appear to suggest that bacterial recombinant MIP-1α may antagonise the inhibitory activity of GI MIP-1α, these results were not statistically significant ($p = 0.17$).

Although these results suggested that the addition of bMIP-1α to sub-inhibitory doses of the bioactive COS 7 MIP-1α may antagonise the bioactivity of the active preparation at a 100:1 molar excess (5%b), these results did not reach statistical significance.

3.2.3. Summary.

Overall these results indicate that the chemokine MIP-1α does not require an accessory factor for its inhibitory activity on keratinocyte proliferation. The selected glycosaminoglycans were unable to activate bacterial recombinant MIP-1α, while COS 7 cells do not appear to produce an accessory factor constitutively or in the presence of bacterial recombinant MIP-1α. Furthermore the dextran transfection process does not appear to stimulate COS 7 cells to synthesise the putative co-factor. Finally although the inactive bacterial protein protein did appear to anatagonise the bioactivity of COS MIP-1α at concentrations reaching approximately a 100:1 molar excess, this result did not achieve statistical significance. Since bMIP-1α is known to bind CCR1, CCR3, CCR5 and CCR9 at the concentrations tested, these results appear to suggest that none of these chemokine receptors are the keratinocyte MIP-1α receptor. The results of the incubation of bMIP-1α with COS MIP-1α however did indicate that if an accessory factor for MIP-1α is present in the latter, it is certainly not present at concentrations in excess of the chemokine concentration.

Moreover overall these results suggest that the COS preparation does not contain an accessory factor for MIP-1α. In order to investigate this further, additional purification of COS MIP-1α was performed.

3.3. Purification.

In order to attempt to understand why GI MIP-1α is active it was decided to attempt to further purify the MIP-1α from the active COS preparation. If a co-factor is present in the active preparation it should be possible to inactivate the MIP-1α by purifying the chemokine to homogeneity. On the other hand if despite this purification of the protein to homogeneity, the chemokine remains bioactive in the KGM assay, this would suggest that COS MIP-1α does not require an accessory factor to inhibit keratinocyte proliferation.

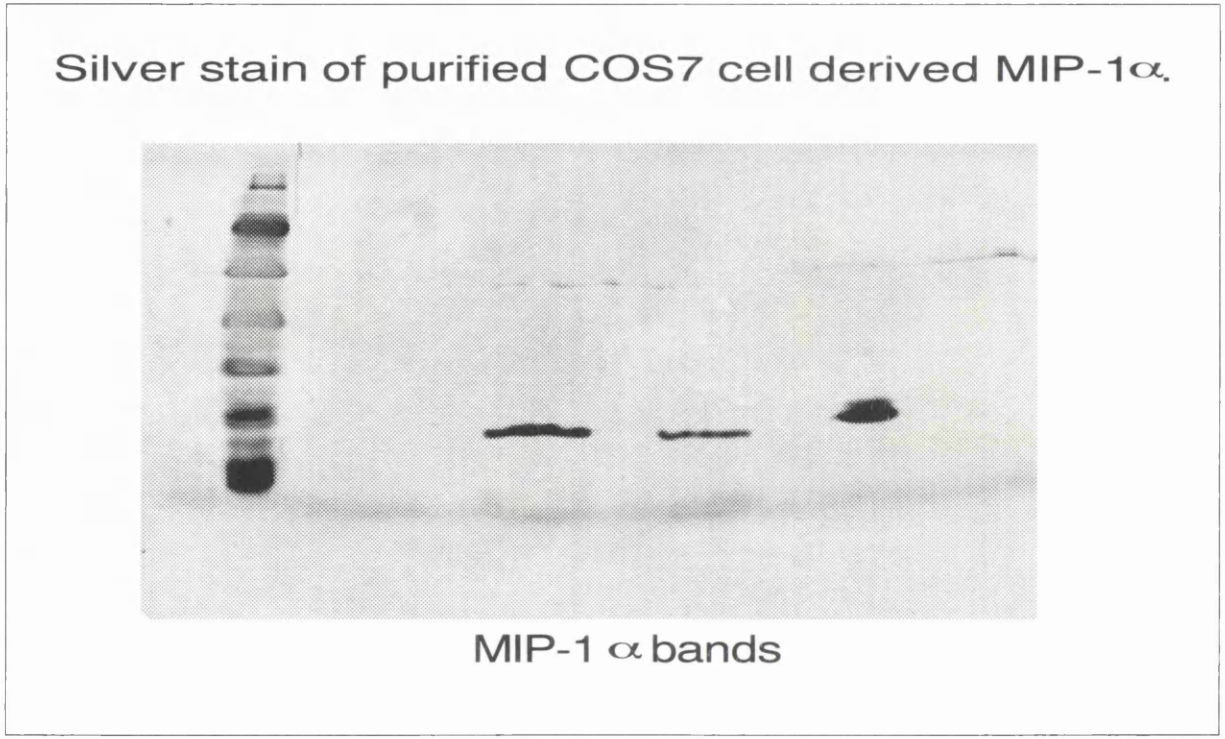
3.3.1. Further purification of GI MIP-1α.

MIP-1α has been previously shown to bind avidly to blue sepharose affinity columns, and this binding affinity has been used to purify MIP-1α (Graham et al 1992). COS MIP-1α was therefore further purified by blue sepharose affinity chromatography. This involved eluting the less strongly binding proteins with 2M NaCl / 0.02M Tris pH 7.6 (fraction B1), and then the most avidly binding proteins with 4.5M $MgCl_2$ (fraction B2). The MIP-1α containing fraction (fraction B2) was then analysed by SDS-PAGE and silver-staining and found to comprise a single band. The silver stain SDS-PAGE gel shown below in *figure 10*, suggests that the B2 fraction is a pure preparation of the chemokine. In order to increase the sensitivity of detection of any contaminating background proteins, the B2 and bacterial MIP-1α preparations were also analysed by autoradiography. Both protein preparations were radiolabelled with ^{125}I and examined on a 15%SDS-PAGE gel by autoradiography. The autoradiographs were exposed for sufficiently long periods of time to ensure a very high sensitivity. The autoradiograph presented in *figure 11 shows* that the B2 preparation is as pure as the commercially available bacterial protein. Even on prolonged exposure no other bands were detected confirming the high level of purity of B2 MIP-1α.

This B2 fraction which is essentially pure MIP-1α in $MgCl_2$ was then desalted into PBS and bioassayed in the KGM assay at a 5% dilution. The controls used for this assay included desalted 4.5M $MgCl_2$, as well as the inactive bacterial MIP-1α. The GI MIP-1α preparation was also used as a control. The results of these assays are presented in *figure 12*. They suggest that B2 is still able to inhibit clonogenic keratinocyte proliferation albeit at a reduced potency. This therefore indicated that a pure form of MIP-1α as determined by both silver staining and autoradiography is still able to inhibit clonogenic keratinocyte proliferation in the KGM assay.

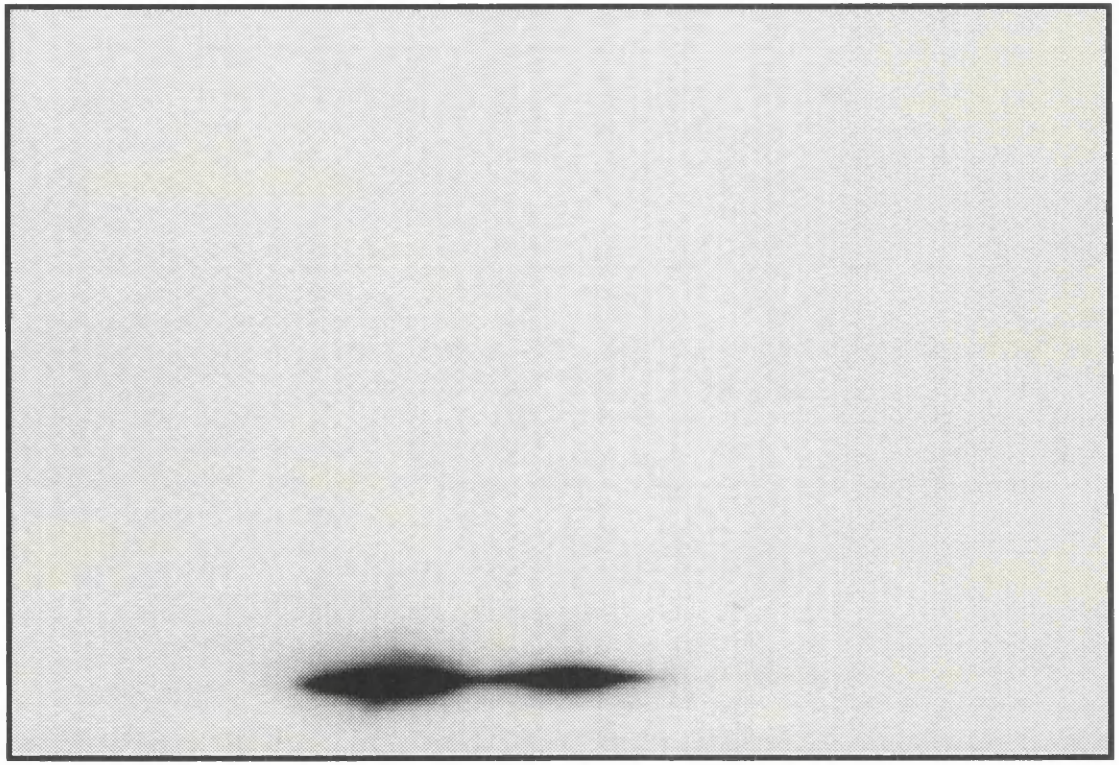
Figure 10.

Silver stain of purified COS 7 MIP-1α.



The above silver stain suggests that B2, which was eluted from a blue Sepharose column in two 1 ml fractions of 4.5M $MgCl_2$, is a pure preparation of MIP-1α.

Figure 11.
Autoradiograph of B2 MIP-1α.

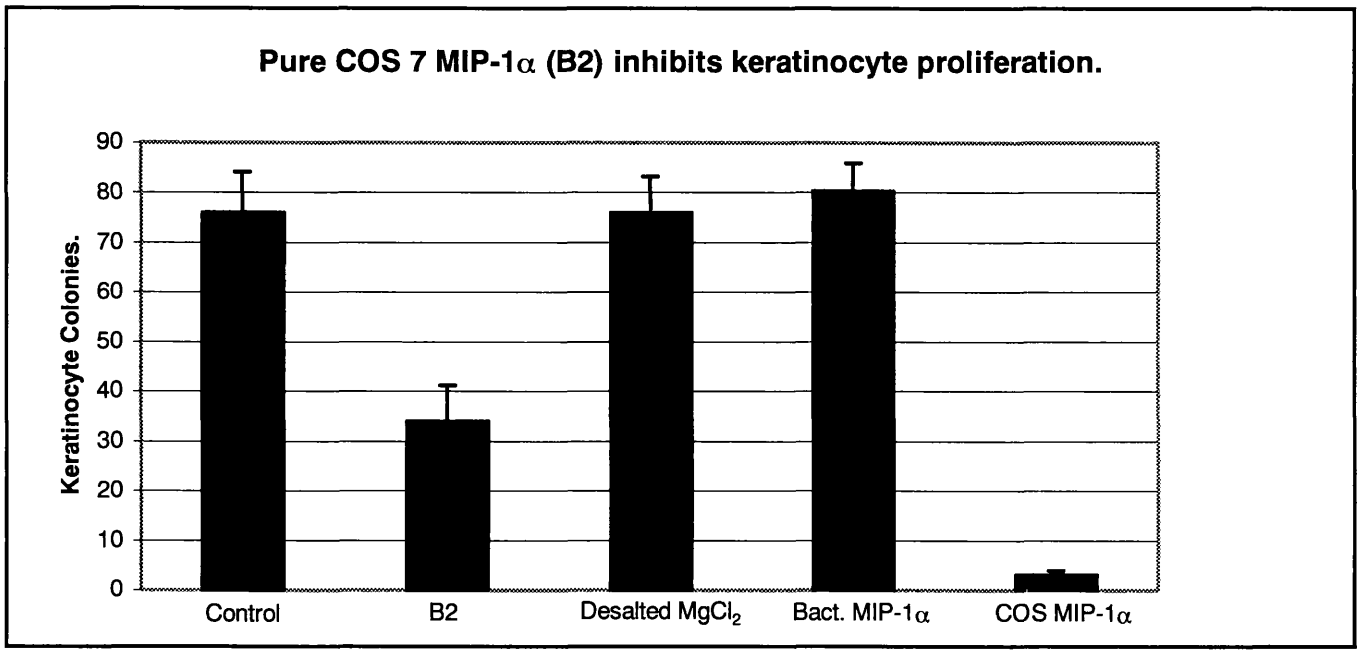


COS 7 Bacterial

The above autoradiograph represents the whole length of a 15%SDS-PAGE gel. Since the 8kDa chemokine runs very close to the dye front, the radiolabelled bands appear at the lower edge of the gel.

Figure 12

B2 is still able to inhibit keratinocyte proliferation.



B2 MIP-1α was assayed at a 5% dilution.
The COS MIP-1α was also assayed at a 5% dilution. The bacterial MIP-1a was assayed at 1μg/ml.

3.3.2.The activity of B2 is reversed by specific anti-MIP-1α polyclonal antibody.

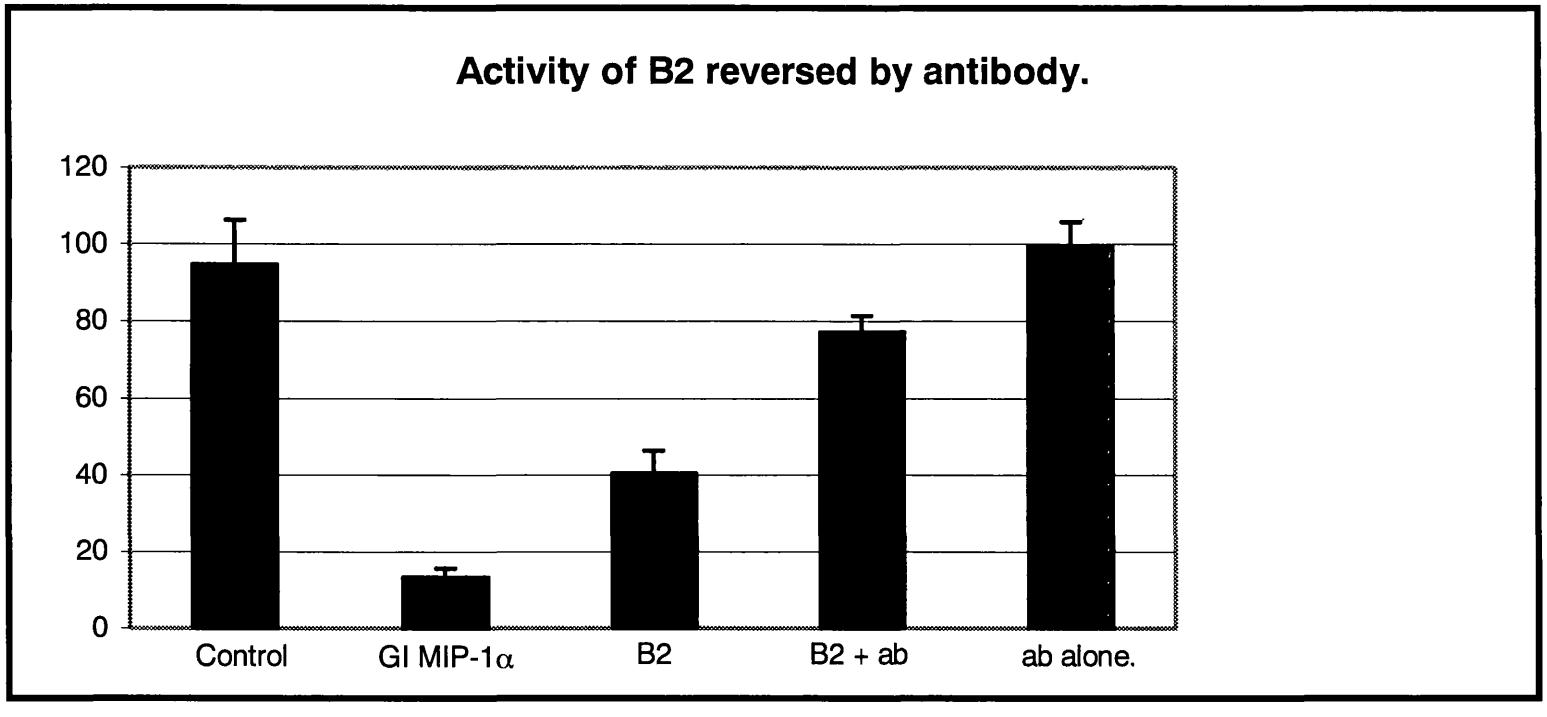
The above results suggest that the pure MIP-1α in the B2 preparation is still able to inhibit keratinocyte proliferation *in vitro*. To further demonstrate that the activity in this purified preparation is due to MIP-1α, specific polyclonal antibody to MIP-1α was added to the B2 preparation for one hour at 37°C. The results of this experiment are presented in *figure 13* and show that the antibody is able to reverse the activity of B2 (This antibody reversal is statistically significant: p = 0.013; paired t-test).

3.3.3. **Why is the B2 MIP-1α preparation not as potent as COS MIP-1α ?**

It is clear from the above that when COS MIP-1α is purified by the combination of heparin and blue sepharose affinity chromatography, this pure form of the protein (B2) is still active in inhibiting the KGM assay, although it is not as potent as the partially pure heparin purifed protein. To elucidate the role of $MgCl_2$ in the inactivation of COS MIP-1α 1 ml of 4.5M $MgCl_2$ was added to 1ml of COS MIP-1α and the mixture was allowed to stand at room temperature for 1hr. A similarly diluted preparation of COS MIP-1α, diluted in phosphate buffered saline, was used as a control. Both preparations were then desalted and assayed in the KGM assay. The pure MIP-1α B2 preparation was also assayed at a similar concentration. The results of these experiment are presented below in *figure 14* . These results suggest that $MgCl_2$ is able to reduce the ability of COS MIP-1α to inhibit clonogenic epidermal keratinocyte proliferation. The reason for this is not entirely clear. However Mg^{2+} is well recognised as being very effective at altering the stability of specific protein conformations. Magnesium is in fact the most potent cation - according to the Hofmeister series - in this ion-protein interaction (T.E. Creighton 1991). This may explain why B2 is less potent than GI MIP-1α. Furthermore Mg^{2+} is also one of the most potent precipitants of proteins and it is also possible that the high salt concentrations used in the protein purification resulted in the "salting out" of the chemokine. It is however clear from these results that Mg^{2+} does reduce the potency of GI MIP-1α on the KGM assay.

Figure 13

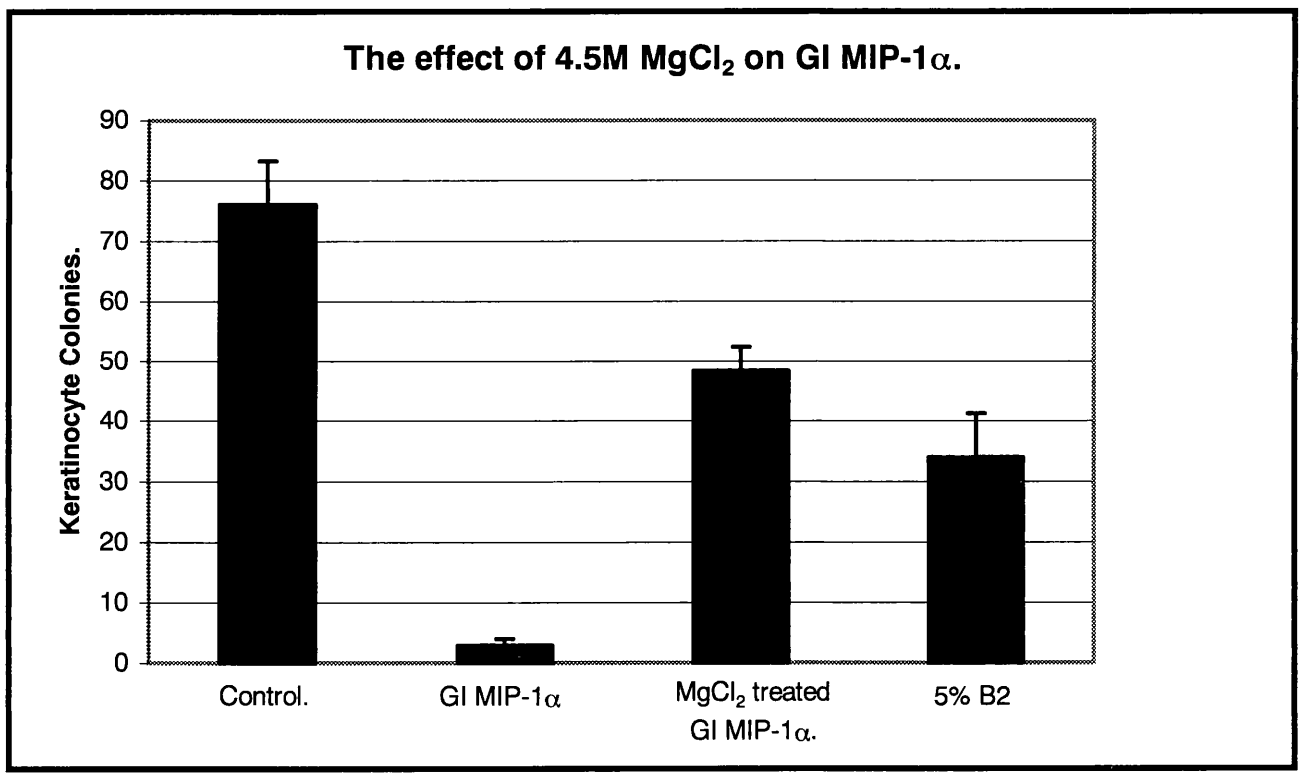
Specific polyclonal antibody to MIP-1α is able to reverse the activity of B2.



GI MIP-1α and B2 were assayed at 5% dilutions.
100μls of specific anti-MIP-1α polyclonal antibody (1mg/ml) was added to the B2 preparation and incubated for 1 hour at 37°C.
The above results show that the specific antibody is able to reverse the activity of B2.
This result was statistically significant (p =0.013; paired t-test).

Figure 14.

4.5M $MgCl_2$ reduces the activity of GI MIP-1α



The control in this assay was 300μls PBS. GI MIP-1α was assayed following a 1:2 dilution in PBS. The $MgCl_2$ treated GI MIP-1α was assayed following a 1:2 dilution in $MgCl_2$. Both protein preparations were then desalted into PBS and assayed at 5% in the KGM assay. B2 was assayed at 5% dilution as an inhibitory control.

3.3.4. **Does B2 contain low levels of other keratinocyte inhibitors?**

The above results strongly suggest that B2 is a pure preparation of MIP-1α. Furthermore they indicate that the keratoinhibitory activity in B2 is due to MIP-1α. It does however remain possible that a very potent synergistic inhibitory factor is present at 'vanishingly' low concentrations in B2. Since MIP-1α has been previously shown to upregulate the expression of the keratinocyte inhibitor TGF-β1 in macrophages (Maltman et al 1993), and since this molecule can inhibit keratinocyte proliferation at low concentrations, the B2 preparation was analysed for the presence of this cytokine. Enzyme-linked immunosorbent assay (ELISA) showed that the total TGF-β1 concentration (both the active and the inactive species) in B2 was less than 5pg/ml (minimum detectable dose). Therefore when B2 is assayed in the KGM assay at a 5% dilution the TGF-β1 concentration in the assay must be less than 250 femtograms/ml or 10 femtomolar. Furthermore other studies have shown that most cell types secrete TGF-β primarily (>90%) as a biologically inactive molecule - in the presence of the latency associated peptide LAP (Miyazono et al 1991). Therefore it is likely that the amount of active TGF-β1 in the B2 preparation is less than 1 femtomolar. These concentrations of TGF-β1 have been previously shown to be unable to inhibit keratinocyte proliferation in this assay, with half-maximal growth inhibition observed at 1ng/ml (Cook et al 1991). Therefore overall these results suggest that TGF-β1 is not contributing to the inhibitory activity in B2. However the above ELISA does not exclude TGF-β2 and TGF-β3. In view of this further experiments were performed to test whether the latency associated peptide (LAP), which reverses the activity of TGF-β 1, 2 and 3, would influence the activity of GI MIP-1α. Unfortunately LAP substantially increases keratinocyte proliferation in the controls by inhibiting autocrine TGF-β making these assays very difficult to interpret since there are now at least three variables in these assays (LAP, endogenous TGF-β and MIP-1α), two of which interact with each other *(figure 15)*. What is clear from this data however is that keratinocyte proliferation in the KGM assay is endogenously regulated by TGF-β. Furthermore since previous experiments have shown that anti-MIP-1α antibody does not result in a similar increase in keratinocyte proliferation in the anti-MIP-1α antibody control assays, the activity in COS MIP-1α is unlikely to be due either wholly or in part to TGF-β.

These results therefore overall suggest that TGF-β is not involved in the inhibitory activity in COS MIP-1α. Furthermore TNF-α was not detected by Western blotting in COS MIP-1α (data not shown). Finally the other known potent keratinocyte inhibitor Epidermal Pentapeptide is unlikely to be present in COS MIP-1α since this preparation has been desalted.

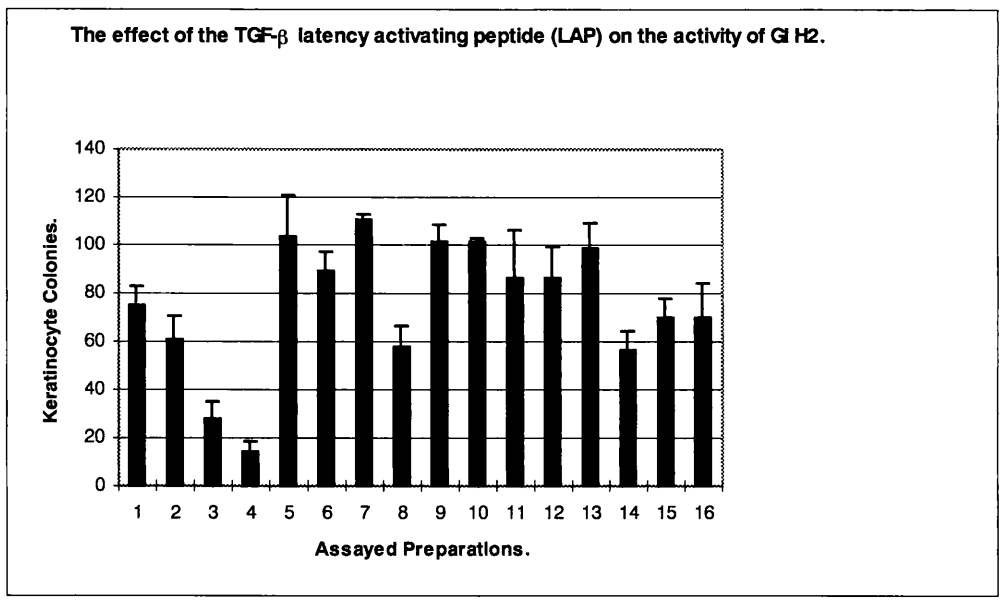
3.3.5. Summary.

These results suggested that a pure form of COSMIP-1α is still able to inhibit keratinocyte proliferation. Moreover this activity was still reversed by anti-MIP-1α antibody. These results suggested that the activity in the COSMIP-1α preparation was wholly due to the chemokine. Nevertheless further studies were performed to ensure that other established potent keratinocyte inhibitors were not present in 'vanishingly' small concentrations in the apparently pure B2 preparation. ELISA analyses suggested that TGF-β1 was not detectable in B2. However KGM assays using the TGF-β Latency Associated Peptide (LAP) were unhelpful since these experiments introduced a separate variable into these assays: keratinocyte derived autocrine TGF-β. Finally TNF-α was not detectable in B2. Also Epidermal Pentapeptide was unlikely to be present in B2 since this material had been prepared for the KGM assay by desalting, which would have removed this small molecule from this preparation.

Coupled with the results presented in the previous section, these findings indicated that COSMIP-1α did not contain an accessory factor, and that the kerato-inhibitory activity of this preparation was pobably due to a activated form of the chemokine. Since there was little objective difference between the inactive bacterial form of MIP-1α and COSMIP-1α on SDS-PAGE gel analyses, it was clear that this activation process had to be the result of some form of subtle structural alteration of the chemokine molecule.

Figure 15.

The effect of the TGF-β latency associated peptide LAP on the activity of GI MIP-1α.



Assayed preparations.

1	Control
2	GI H2 1%
3	GI H2 3%
4	GI H2 5%
5	LAP 50 ng (to 6mls KGM)
6	LAP 100 ng
7	LAP 250 ng
8	GI H2 1% + LAP 50 ng
9	GI H2 1% + LAP 100 ng
10	GI H2 1% + LAP 250 ng
11	GI H2 3% + LAP 50 ng
12	GI H2 3% + LAP 100 ng
13	GI H2 3% + LAP 250 ng
14	GI H2 5% + LAP 50 ng
15	GI H2 5% + LAP 100 ng
16	GI H2 5% + LAP 250 ng

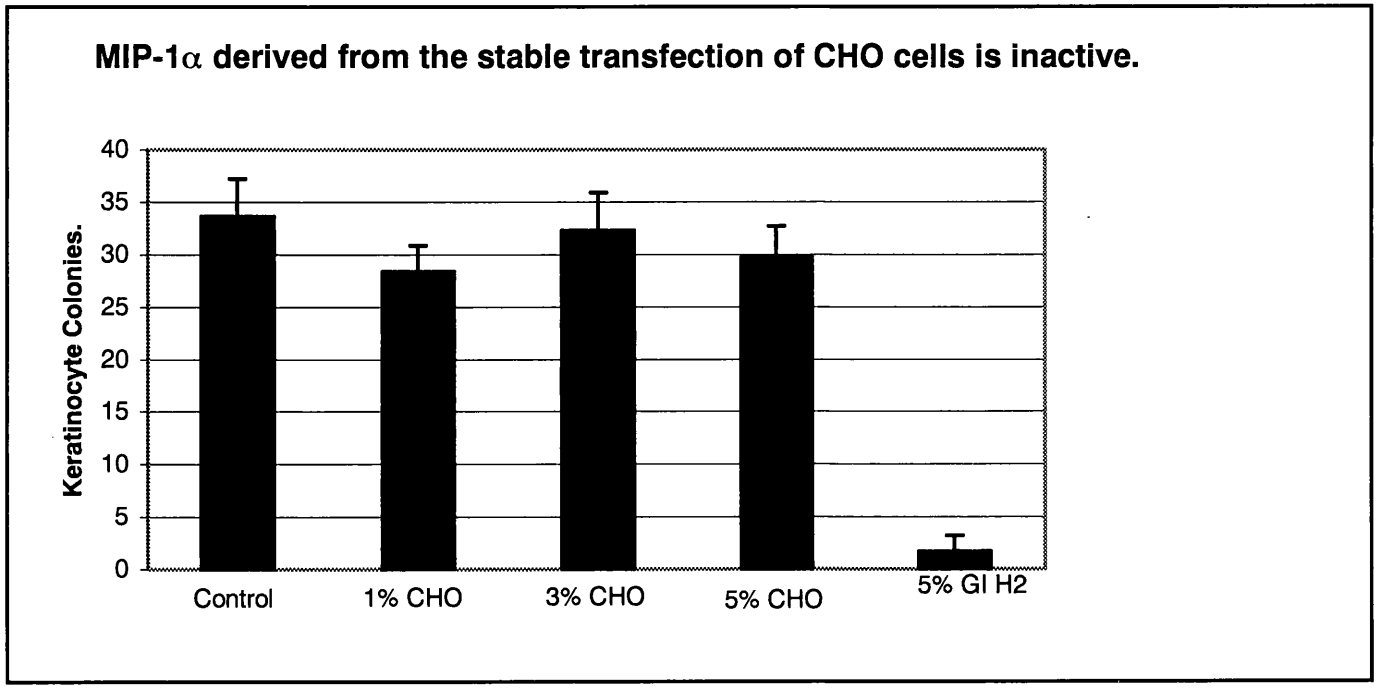
GI MIP-1α (GI H2) inhibition in the above (lanes 2-4) follows a dose-response relationship which is statistically significant (p=0.008)..
LAP increases keratinocyte proliferation (5-7). This is statistically significant (p=0.019).
The effects of GI MIP-1α on the KGM assay in the presence of LAP are difficult to interpret and overall not statistically significant (8-16).
The only other statistically significant result (p=0.006) in this series of experiments is the increase in KGM colony formation that LAP induces in the presence of 3% GI MIP-1α (ie comparing lane 3 and lane 13). This is however likely to be related to the inhibition of endogenous keratinocyte derived TGF-β rather than the reversal of TGF-β in the GI MIP-1α preparation (cf statistically significant rise in colony formation between lanes 1 and 7).

3.4. The activity of human MIP-1α derived from a stably transfected CHO cell line .

The above results demonstrate that while MIP-1α is secreted in an active form by the mammalian COS 7 cell expression system, the prokaryotic bacterial expression system secretes a protein that is inactive on keratinocytes. Since bacteria lack some of the post-translational regulatory mechanisms present in mammalian cells, we postulated that the COS 7 cell expression system may be able to secrete a functionally active MIP-1α protein as a result of some form of structural alteration. To investigate this further we tested the activity of MIP-1α generated from a different mammalian cell, a stably transfected chinese hamster ovary (CHO) cell line. If CHO cell derived MIP-1α was active in the KGM assay, this cell line would prove to be a very useful source of MIP-1α for further analyses of the activation process.

This CHO cell line which was stably transfected to express human MIP-1α (LD78β) was a gift from Genetics Institute (Mass., USA). This cell-line is cultured in the presence of methotrexate to select for the stably transfected cells as already described in the methods section. When supernatant containing MIP-1α was required, the CHO cells were washed twice with serum-free Special Liquid Medium (Gibco) and the cells were cultured for 72 hours. The supernatant was then collected and analysed by Western blotting for the presence of MIP-1α. The supernatant was then partially purified by heparin affinity chromatography, desalted and analysed for the presence of MIP-1α by SDS-PAGE gel electrophoresis followed by Western-blotting or silver-staining. This CHO cell derived protein was then bioassayed in the clonogenic keratinocyte assay at 1%, 3% and 5% dilutions of a 10μg/ml preparation. The results of these assays are presented in figure 16 and are representative of three separate assays. The CHO cell MIP-1α did not significantly inhibit keratinocyte proliferation. Furthermore this human CHO cell derived MIP-1α has been previously shown in our laboratory to be active on the murine haemopoietic CFU-A assay, confirming the biological activity of this chemokine preparation. These results appear to suggest that not all eukaryotic cells are able to secrete a form of MIP-1α that is able to inhibit keratinocyte proliferation in the KGM assay. The alternative explanation is that huMIP-1α, unlike muMIP-1α, does not inhibit keratinocyte proliferation.

Figure 16.
CHO cell human MIP-1α is inactive.



The heparin purified GI MIP-1α (GI H2) was assayed at a 5% dilution.
The heparin purified CHO cell MIP-1α was assayed at 1%, 3%, 5% dilutions.
CHO cell MIP-1α was inactive on the KGM assay at all the concentrations tested

3.5. Does some form of structural alteration activate MIP-1α ?

The above results have shown that murine COS MIP-1α is able to inhibit clonogenic keratinocyte proliferation in a very pure form as defined by the presence of a single band on both a silver stained SDS-PAGE gel and an autoradiograph of ^{125}I labelled protein. This and the previous results have suggested that MIP-1α is activated by some form of subtle structural alteration. There is a precedent in the chemokine family for activation by structural alteration. For example the neutrophil activating CXC chemokine NAP-2 is derived from the cleavage of the full-length peptide Platelet Basic Protein (PBP), which has little activity on neutrophils (Walz and Baggiolini 1989). Another CXC chemokine PF-4 has recently been shown to be secreted by activated human peripheral blood leukocytes in an amino-terminal truncated form which is fifty times more potent than the full-length protein in inhibiting the proliferation of endothelial cells (Gupta et al 1995). It was therefore postulated that in order to inhibit keratinocyte proliferation, MIP-1α may need to be activated through some form of structural alteration.

3.5.1. Protein Sequencing.

Since the purified form of COS MIP-1α (B2) was still able to inhibit human epidermal clonogenic keratinocyte proliferation, it was decided to analyse the MIP-1α protein present in this preparation by Edman degradation protein sequencing. The bacterial recombinant murine MIP-1α protein obtained from R&D systems was known to have the wildtype amino-terminal sequence *(APYGADTPT...).* The COS 7 cell derived protein preparation was also shown to primarily comprise the wildtype full-length protein like the bacterial recombinant cytokine. However the sequencing reaction also suggested the presence very low concentrations of a -4 amino-acid truncated variant of MIP-1α (ADT ...).

Full-length murine MIP-1α: APYGADTPTACC.....

- 4 murine MIP-1α variant: ADTPTACC.....

Interestingly when one of the human variants of MIP-1α, LD78, was initially sequenced (Obaru et al 1986) the signal peptidase cleavage site was initially thought to be at position -4 and the wildtype protein was thought to be what is now thought to be a -4 mutant. The

presence of the detected -4 mutant in the COS 7 supernatant may therefore be related to the presence of an alternative signal peptidase cleavage site. Interestingly further study of the MIP-1α sequence revealed that in fact this -4 position may potentially function as a signal peptidase cleavage site (Von Heijne 1986; Von Heijne personal communication). This -4 variant may therefore be an alternative product of signal peptidase cleavage.

Human MIP-1α /LD78 (Obaru et al 1986): **_____ADTPTACC....**

(Full-length equivalent of murine protein: **APLAADTPTACC.....)**

There is some evidence in the literature that MIP-1α can be secreted as a -4 truncated mutant. Studies investigating human immunodeficiency virus (HIV) suppressive factors have suggested that an immortalised human CD8+ T lymphocyte cell line secretes a -4 truncated variant of human MIP-1α (Cocchi et al 1995). Furthermore this -4 human MIP-1α protein (ADTPTACCFSYTSR.....) was the only MIP-1α variant detected (secreted at 615ng/ml). The authors therefore suggested that this sequence may in fact correspond to the actual human T-lymphocyte MIP-1α amino-terminus. However this is different to previously suggested (but not sequenced) published sequences of LD78 (Nakao et al 1990; Schall et al 1991).

Nakao et al (1990) **APLAADTPTACC... (LD78β)**

Nakao et al (1990) **ASLAADTPTACC... (LD78α)**

Schall et al (1991): _SLAADTPTACC...

Rollins et al (1991): ASLAADTPTACC..

It therefore is clear that there has been some confusion in the literature regarding the amino-terminal sequence of the mature human MIP-1α protein. As has already been described in the introduction, Nakao et al (1990) suggested that there are at least two non-allelic human MIP-1α genes: LD78α and LD78β. The amino acid sequences of these two genes vary in only 5 amino-acids, with two of these amino-acid changes being conservative, reciprocal serine to glycine changes. However the other 3 amino-acid changes are clustered around the signal peptidase cleavage site. Nakao et al (1990) suggested that these two homologous

human MIP-1α genes probably result in identical signal peptide cleavage sites to create nearly identical 69 amino-acid molecules.

Figure 17.

LD78α and LD78β amino-acid sequences including signal peptide.

LD78α

MQVSTAALAVLLCTMALCNQF*SASLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA

LD78β

MQVSTAALAVLLCTMALCNQVLSAPLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPSVIFLTKRGRQVCADPSEEWVQKYVSDLELSA

The 5 amino-acids differences between LD78α and LD78β are underlined.

The mature proteins LD78α and LD78β according to Nakao et al (1990).

LD78α: **ASLAADTPTACC...**

LD78β: **APLAADTPTACC...**

However on closer inspection of the two different signal peptidase cleavage sites, if Von Heijne's rule for the prediction of signal sequence cleavage is followed (Von Heijne 1986), these two genes result in different signal peptidase cleavage sites. While LD78α should be secreted as a -4 amino-terminally truncated 65 amino-acid protein (Von Heijne's -3, -1 rule indicates that the amino-acid glutamine (Q) should not be present at position -3 in the signal sequence), LD78β should be secreted as a 69 amino acid molecule.

LD78α: **____ADTPTACC...**

LD78β: **APLAADTPTACC...**

This has not however been previously defined in the literature, probably because it is very difficult to separate such homologous proteins of this size on an SDS-PAGE gel or by HPLC. However Nakao et al (1990) showed that both LD78α and LD78β mRNAs are transcribed. Furthermore the findings of Cocchi et al (1995) suggest that a -4 protein is secreted.

Interestingly similar findings have previously been observed with other chemokines. In fact sequence analysis of IL-8 has shown that not only does it occur in natural mixtures of multiple forms that differ in truncation at the amino-terminus, but that the composition of IL-8 preparations depends on the cell source used to produce it rather than on the purification method (Van Damme 1990; reviewed by Van Damme 1991). However the variability in the

amino-terminus of these chemokines is probably not all related to different signal peptidase cleavage sites.

The above results therefore suggest that MIP-1α may be secreted in different amino-terminal truncated forms. Moreover recent work in this laboratory has shown that these different amino-terminal variants of MIP-1α have very different chemokine receptor-binding affinities (R.J. Nibbs and G.J. Graham personal communication). We therefore postulated that murine MIP-1α may be activated by the mammalian COS 7 cells into a keratinocyte inhibitory peptide by amino-terminal truncation.

3.5.2. Amino-terminal truncated mutants.

The human form of MIP-1α, LD78, was initially identified as a mRNA induced in tonsillar lymphocytes. As already discussed above, the gene was thought to secrete a protein that is a -4 variant of the full-length protein (Obaru et al 1986). This -4 truncated amino-acid sequence has previously been used in our laboratory to generate non-aggregating human mutants of MIP-1α. A bacterial recombinant -4 amino-terminally truncated mutant form of LD78 or human MIP-1α was therefore available in our laboratory. This non-aggregating mutant has three amino-acid substitutions at the carboxy terminus which neutralise specific acidic residues, as discussed in section 1.3.3.3.

Full-length hu MIP-1α: **APLA**ADTPTA CC FSY..... C ADPSEEWVQ**K**YVS**D**L**E**LSA
-4 hu MIP-1α mutant 1(M1): ADTPTA CC FSY..... C ADPSEEWVQ**Q**YVS**N**L**Q**LSA*
**The mutations at the carboxy-terminus were generated to result in a non-aggregating mutant.*

This non-aggregating truncated mutant M1 forms dimeric aggregates and has been shown to inhibit proliferation of haemopoietic stem cells in the CFU-A assay with a potency that is indistinguishable from that of the wildtype molecule. This truncated protein was therefore analysed in the KGM assay. Bovine serum albumin (BSA from Sigma Ltd) was used as a carrier protein in these experiments to minimise chemokine loss (0.5 mg/ml BSA). BSA alone, at the concentrations used, had no effect on keratinocyte proliferation. The results of these assays are presented in *figure 18* and show that the amino-terminally truncated form of hu MIP-1α M1, unlike the full-length protein, is able to inhibit keratinocyte proliferation in the KGM assay. Furthermore this activity is reversible by a specific polyclonal antibody to

human MIP-1α (figure 19). The full-length human MIP-1α molecule is inactive when analysed in this assay at concentrations up to 10μg/ml. Furthermore the murine non-aggregating mutant proteins, which have a full-length amino-terminus were also found to be inactive. This suggests that the activity seen in the truncated human M1 is related to the N-terminal truncation and not the non-aggregating state.

However although the kerato-inhibitory activity of this amino-terminally truncated form of MIP-1α was confirmed in several assays, there appeared to be significant variability in the kerato-inhibitory activity of various batches of truncated protein. The cause of this variability was not clear in these experiments, but may have been related to repeated freeze-thawing. However it was clear that some batches of the bacterial amino-terminally truncated preparations were more active than others. Further analysis of COS 7 MIP-1α using electrospray mass spectroscopy was therefore performed to attempt to determine the molecular mass of any truncated form of kerato-inhibitory MIP-1α in this preparation.

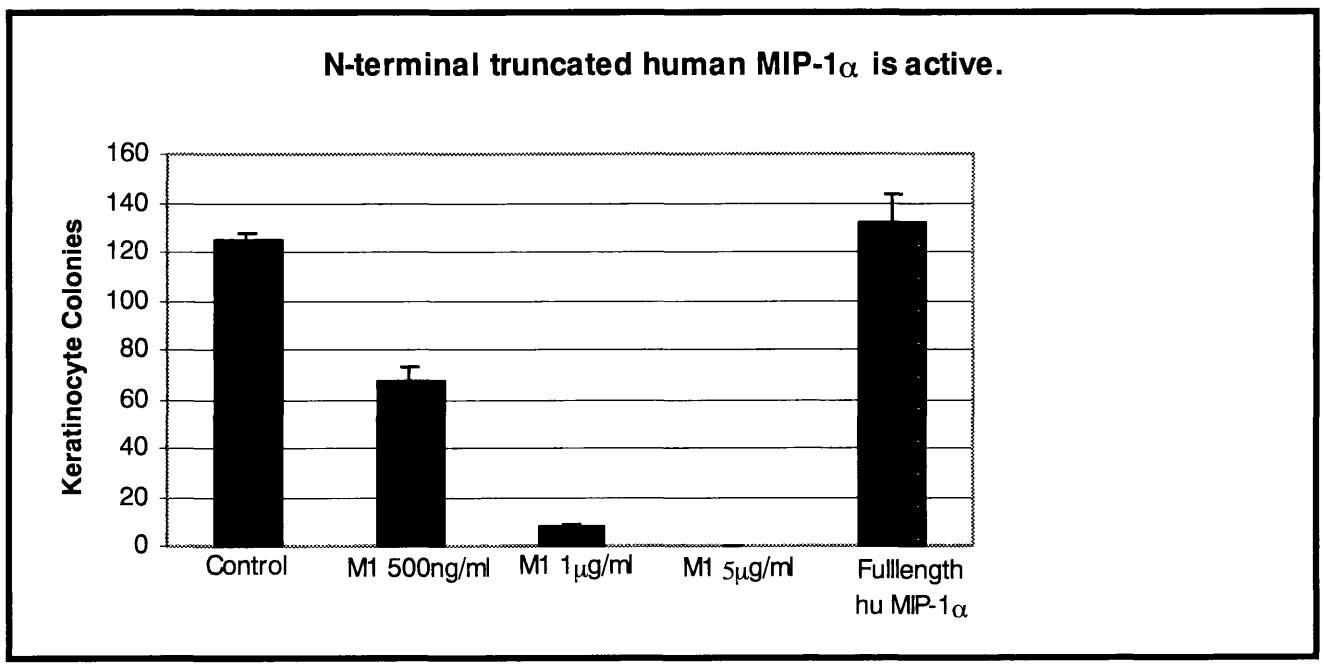
3.5.3. Electrospray Mass Spectroscopy.

The mass of a protein is a definitive and informative parameter that is now experimentally accessible as a result of the recent technological advances in the field of mass spectrometry. Electrospray mass spectroscopy (EMS) is able to accurately determine the molecular weight of proteins and polypeptides with picomolar sensitivity (Siuzdak 1994). A number of studies have used EMS to determine structural modifications in proteins that result in minor mass differences that are not detectable by the standard methods of protein analysis. In order to determine the mass or molecular weight of the purified COS 7 cell derived MIP-1α (B2 fraction) this active preparation was analysed by EMS with the help of Dr. Tino Krell (Department of Biochemistry, Glasgow University). The EMS of MIP-1α was considerably complicated by the aggregating nature of the chemokine. This was partly abrogated with the use of 0.1% acetic acid. The results obtained are presented in *figure 20.* They suggested that the B2 preparation is made up of a mixture of different MIP-1α variants which differ at their amino-terminal end. The wildtype protein appeared to be the main species. However a number of other amino-terminally truncated species were also detected with molecular weights of 7805, 7716, 7559, 7500, 7422, 7217 and 7142. These species are likely to correspond to -1 MIP-1α (calculated mass 7813), -2 MIP-1α (calculated mass 7716),

-3 MIP-1α (calculated mass 7553), -4 MIP-1α (calculated mass 7496), -5 MIP-1α (calculated mass 7425) and -7 MIP-1α (calculated mass 7209) and probably a variant of the -8 protein (calculated mass 7112).

Figure 18.

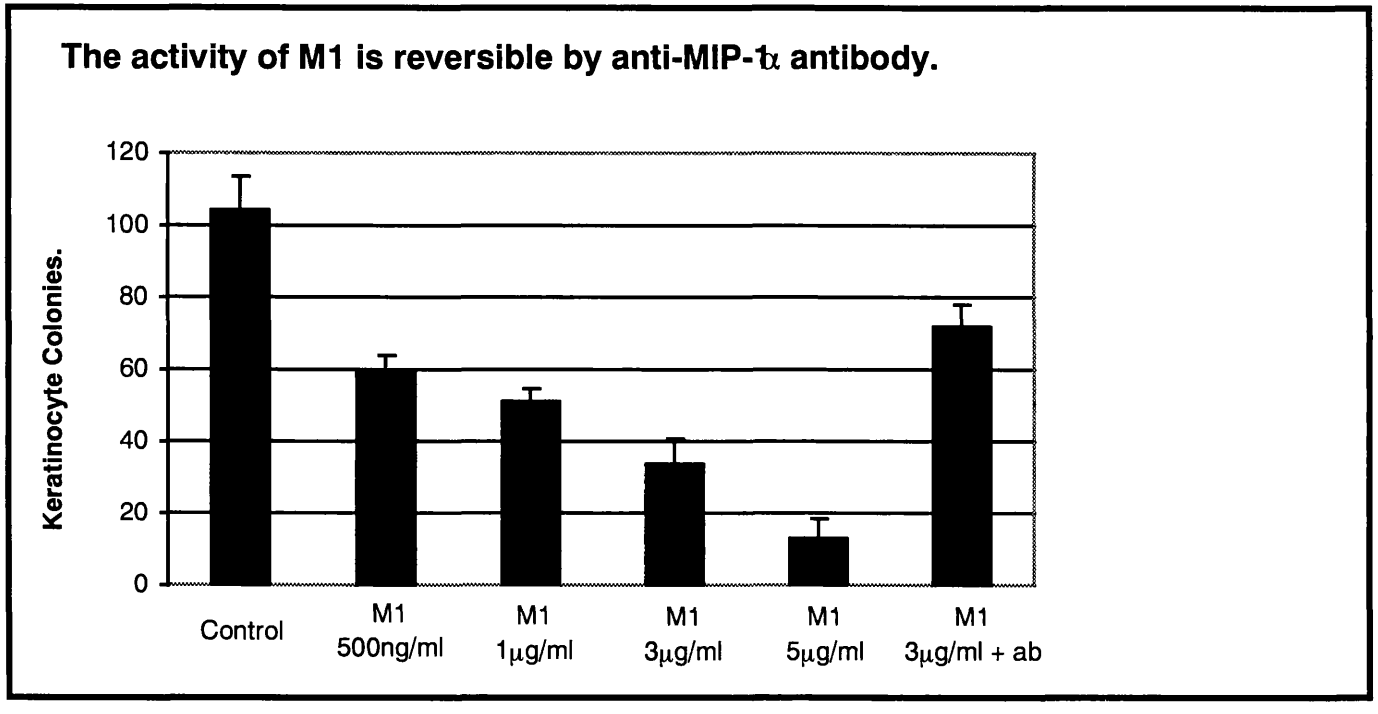
The -4 amino-terminal truncated, non-aggregating, bacterial recombinant form of human MIP-1α (M1) is active.



M1: -4 amino-terminally truncated non-aggregating human MIP-1α mutant (dimeric form).
Full-length human MIP-1α was assayed at 5μgs/ml (CHO cell MIP-1α).
M1 was active on the KGM assay with an ED50 of approximately 500ng/ml.

Figure 19.

The activity of M1 on the KGM assay is reversible by a specific anti-human MIP-1α antibody.



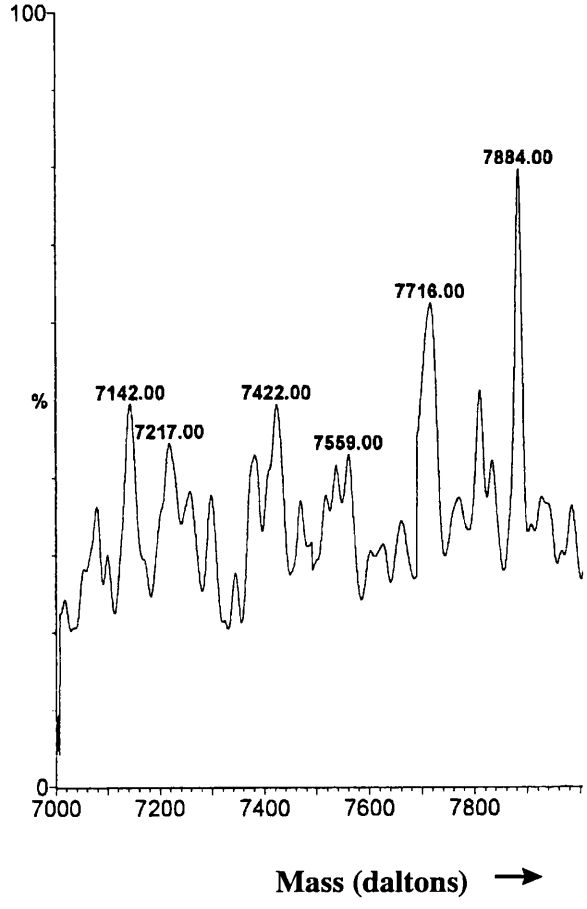
Both M1 and the polyclonal anti-MIP-1α antibody were obtained from R&D systems.
The neutralisation reaction was carried out at 37 ^{0}C for one hour.
This antibody reversal is statistically significant (p =0.005; paired t-test).

Figure 20

Electrospray Mass Spectroscopy of purified COS 7 MIP-1α.

Calculated molecular masses.

Wildtype	**7884**
-1	**7813**
-2	**7716**
-3	**7553**
-4	**7496**
-5	**7425**
-6	**7310**
-7	**7209**
-8	**7112**



3.5.4. Activation by proteolytic cleavage.

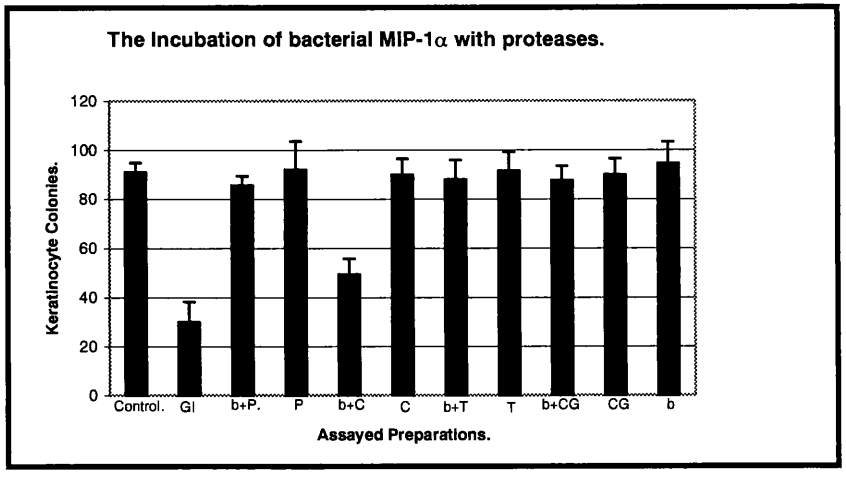
The above results appeared to indicate that an amino-terminally processed MIP-1α species is the keratinocyte-inhibitory species, however the variability we had observed in the activity of the -4 non-aggregating mutant was concerning. In order to study this activation process further it was decided to investigate whether bMIP-1α can be activated by proteolytic cleavage of the amino-terminus using proteases with recognition sites in this area. It had been previously shown that the active truncated CXC chemokine NAP-2 is formed by the proteolytic cleavage of CTAP-III by chymotrypsin and cathepsin G (Car et al 1991). The inactive full-length bacterial recombinant MIP-1α (b) was therefore incubated with cathepsin G (CG at 0.25μg/ml), agarose bound chymotrypsin (C at 0.25μg of protein/ ml), plasmin (P at 1U/ml) and thrombin (T at 1U/ml) and then analysed for kerato-inhibitory activity. Approximately 5μg of murine MIP-1α was incubated with each of the above proteases. The proteases were all incubated for approximately 1 hour at 37°C in phosphate-buffered saline pH 7.4 as previously described (Car et al 1991). Parallel control reactions were also set up without MIP-1α. Furthermore after the chymotrypsin incubation the reaction was microcentrifuged and then filtered through a 0.2μ filter to remove the agarose bound chymotrypsin.

The reactions were then all diluted by addition to 6mls of keratinocyte containing KGM and bioassayed. The results, which are presented below in *figure 21* show that none of the control reactions comprising only protease were able to inhibit keratinocyte proliferation. Plasmin has been previously shown to activate TGF-β by cleaving LAP from the inactive parent molecule, therefore an increase in keratinocyte colony formation was anticipated. However the resulting plasmin concentration in these KGM assays (< 0.1U/ml) was not high enough to be active on TGF-β (1-2U/ml) as shown by Lyons et al (1990) .

Furthermore bMIP-1α was not activated by cathepsin G, plasmin or thrombin. However chymotrypsin was able to generate a weak keratinocyte inhibitory form of MIP-1α (statistically significant p= 0.014; paired t-test). The chymotrypsin control had no detectable effect on keratinocyte proliferation.

Figure 21.

Does proteolytic cleavage activate bacterial recombinant MIP-1α?



Control	300 µls of PBS
GI	300µls of GI MIP-1α.
B+P	Bacterial MIP-1α incubated with plasmin
P	Plasminogen alone
b+C	Bacterial MIP-1α incubated with chymotrypsin
C	Chymotrypsin alone
b+T	Bacterial MIP-1α incubated with thrombin
T	Thrombin alone
b+CG	Bacterial MIP-1α incubated with cathepsin G
CG	Cathepsin G alone
b	Bacterial MIP-1α alone

The inhibitory activity of the chymotrypsin treated bMIP-1α is statistically significant (p= 0.014; paired t-test).

This result suggested that like the CXC chemokine CTAP-III, the inactive bacterial recombinant murine MIP-1α can be converted into a weakly active MIP-1α species by chymotrypsin proteolytic cleavage. None of the other three proteases used were able to activate the chemokine. Since chymotrypsin cleaves peptide bonds at the carboxyl end of tyrosine, phenylalanine and tryptophan. It was therefore possible that truncation of MIP-1α at the very accessible amino-terminal tyrosine at position 3 was activating the chemokine. However other sites of proteolytic activation could not be ruled out.

3.5.5. Summary

These results suggested that amino-terminal processing may convert the MIP-1α molecule into an inhibitor of keratinocyte colony formation. However this truncated form of human MIP-1α mutant M1 did not appear to be as active in inhibiting cells in the KGM assay as GI MIP-1α. The reason for this discrepancy may be that one of the other variants detected by EMS is considerably more potent than this -4 amino-terminally processed form. It was interesting to note at this point that the MIP-1α amino-terminus contains a near-perfect cleavage site for the Interleukin-1β Converting Enzyme (ICE) family of proteases. The four amino-acid motif YGAD at positions -3 to -6 in the MIP-1α amino-terminus is very similar to the YVAD motif recognised by ICE (Sleath et al 1990; Wilson et al 1994). ICE cleaves at the carboxyl end of the aspartate in this motif. Although COS 7 cells do not appear to express ICE (Ceretti et al 1992), epidermal Langerhans cells express functional ICE (Ariizumi et al 1995). Furthermore ICE knockout mice appear to have a defect in the export of a number of cytokines other than IL-1β (Kuida et al 1995). Processing by ICE appeared to be a potential mechanism by which MIP-1α could be converted into an inhibitor of keratinocytes..

Figure 22.Proteolytic processing by Interleukin-1β Converting enzyme.

Recognised ICE cleavage sites (....ASP - X....) include:

...YVAD⇓X

...YVHD⇓A

...YVHD⇓G

...YVFD⇓A

...LSAD⇓G

**Where X is any amino-acid (Wilson et al 1994).*

***Although AYVHD⇓AP... is cleaved, HDAP... is not (Sleath et al 1990).*

When both the human and murine MIP-1α amino-termini are examined potential ICE cleavage sites are identified:

Murine MIP-1α:

APYGAD⇓TPTA...

Murine MIP-1α is likely to be a substrate for ICE (Sleath et al 1990; Wilson et al 1994; Ramage et al 1995).

Human MIP-1α (LD78β but not LD78α: see section 1.3.3.1):

LD78α mature amino-terminus: ADTPTA....

LD78α is not likely to be an ICE substrate: (see above: Sleath et al 1990).

LD78β mature amino-terminus: APLAAD⇓TPTA....

LD 78β is likely to be cleaved by ICE (Sleath et al 1990; Wilson et al 1994; Ramage et al 1995)

3.6. Reverse Phase Chromatography.

The above results suggest that GI MIP-1α contains low concentrations of amino-terminally truncated forms of this chemokine. Therefore in order to identify the kerato-inhibitory species it was decided to attempt to purify the active form of MIP-1α by reverse phase chromatography. GI MIP-1α was therefore purified by reverse phase chromatography as shown in *figure 23*. The protein preparation was eluted from a reverse phase column by increasing concentrations of acetonitrile/ 0.1%TFA. All the fractions eluted were assayed in the KGM assay. The results of these assays are presented in *figure 24* and indicate that the most active fractions were fractions 19, 20 and 21 (figure 24). This was surprising since fraction 19 did not appear to have any detectable MIP-1α although this fraction did appear to have an approximately 70kDa band (see figure 25). Further analysis revealed that the most potent active fraction was fraction 20 (figure 26). Furthermore the activities of all these active fractions (19, 20 and 21) were reversible with specific goat polyclonal antibody to MIP-1α (figure 27). These results show that the MIP-1α concentration does not define the kerato-inhibitory activity of the reverse phase fractions 19, 20 and 21. Furthermore it is clear that in fraction 19 there is no detectable MIP-1α on the silver-stained SDS-PAGE gel, although the activity of this fraction is fully reversible by anti-MIP-1α antibody.

The identity of the 70kDa band in fraction 19 is not known. We decided to investigate whether this 70kDa protein is able to potentiate the activity of MIP-1α. We postulated that since MIP-1α is present at very low concentrations in fraction 19, if this 70 kDa band potentiates the activity of the chemokine, the addition of bacterial recombinant MIP-1α to this preparation should increase the activity of fraction 19. The results of this assay are presented in *figure 28* and show that bacterial recombinant MIP-1α does not increase the activity of *fraction 19*. On the other hand these results suggest that bMIP-1α may in fact decrease the activity of fraction 19. The mechanism by which this occurs is not clear but it may be due to direct competition of high levels of bMIP-1α for receptor binding or perhaps indirectly as a result of MIP-1α aggregation leading to the sequestering of the active MIP-1α mutant. This effect on the active MIP-1α molecule (see *figure 9*) has only been observed at very high relative concentrations of bMIP-1α to the active molecule, suggesting that the full-length molecule is a poor antagonist of the active species. What is however clear is that these results indicate

that the observed 70kDa band is not an accessory factor for MIP-1α. Moreover since the activity of fraction 19 is fully reversible by the specific polyclonal antibody to the chemokine these results suggest that the active form of MIP-1α in this preparation is very potent. Since the chemokine is not detected in fraction 19 on a silver-stained SDS-PAGE gel or Western blot the active form of the chemokine is probably active at picomolar concentrations. Furthermore it is clear that this active form of the chemokine must be present in the COS 7 cell preparation at very low concentrations.

The active RPC fractions 19, 20 and 21 were analysed further by Edman degradation protein sequencing. However no sequence was detected in fraction 19 and only the full-length MIP-1α sequence was detected in fractions 20 and 21. ESM spectroscopy of these samples encountered similar difficulties and no molecular weight was detected in fraction 19. These results may however be partly a result of chemokine loss and protein dilution during the chemokine purification process.

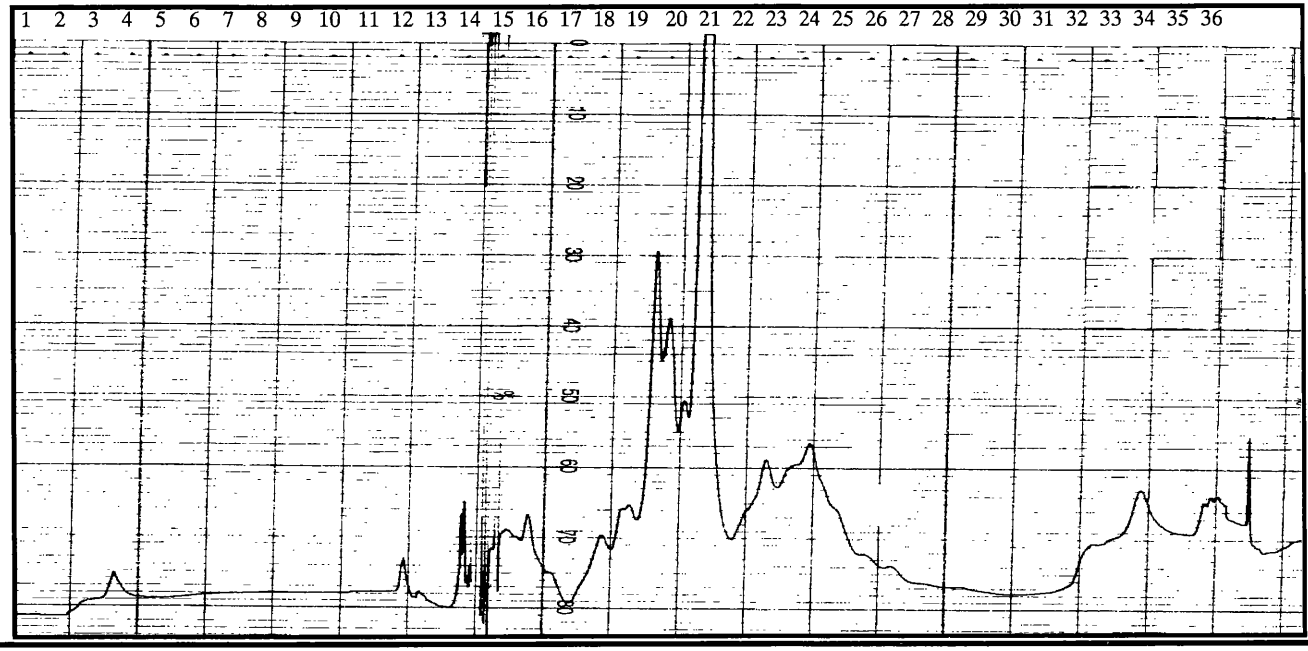
3.6.1. Summary.

Overall these results suggested that COS MIP-1α comprises mainly the inactive form of MIP-1α, the active form comprising only a small proportion of the chemokine concentration. These results also suggested that the active form of MIP-1α is very potent, probably being active at picomolar concentrations. These results also appeared to indicate that the kerato-inhibitory activity may be due to a structurally altered form of MIP-1α. It was postulated that in light of our electrospray data on the B2 preparation, and the activity of the -4 amino-terminally truncated mutant M1, that the chemokine is activated by amino-terminal truncation. It was decided that the best way to investigate this further was the generation of amino-terminally truncated MIP-1α mutants in order to assess their activity in the KGM assay.

Figure 23.

Reverse Phase Column (RPC) Chromatography run of GI MIP-1α.

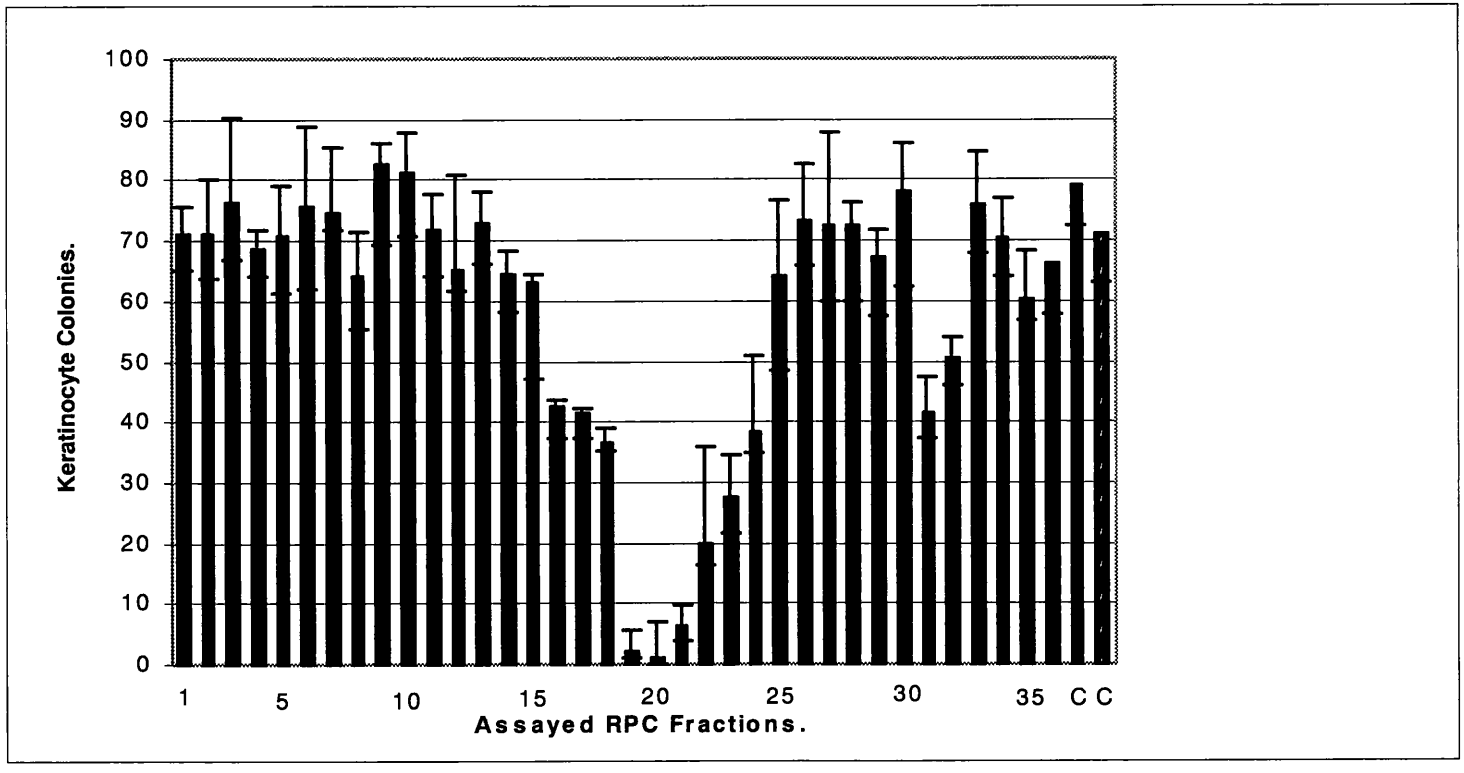
Eluted 1ml Fractions (fractions 1 to 36).



10mls of GI MIP-1α was purified by reverse phase chromatography(RPC) using a 1ml Pharmacia Resource reverse phase column. The protein preparation was eluted off the column by by a variable gradient of acetonitrile/ 0.1%TFA.. After loading the column with GI MIP-1α 10 mls of 0% acetonitrile/ 0.1%TFA was employed to elute any non-binding proteins (fractions 1-10). A gradient of 0 to 30% acetonitrile was used over the next 5mls (fractions 10-15). (This resulted in a large peak at fraction 14 which contained no detectable protein or KGM inhibitory activity. This peak was routinely seen with RPC purifications and may contain PBS derived contaminants). Between fractions 15 and 30 a shallower gradient was employed (30-70% over 15mls) since pilot RPC purifications using bMIP-1α had shown that the bacterial recombinant chemokine eluted as a sigle peak at ~44% acetonitrile/ 0.1%TFA. The RPC purification of GI MIP-1α resulted in a number of major peaks around this point (fractions 19, 20 and 21). The RPC run was then completed by a steep gradient of 70-100% acetonitrile/ 0.1% TFA. All the fraction were collected as 1ml volumes and then dried by "speedivac" and redissolved in PBS. Each fraction was then analysed by SDS-PAGE and assayed on the KGM assay.

Figure 24

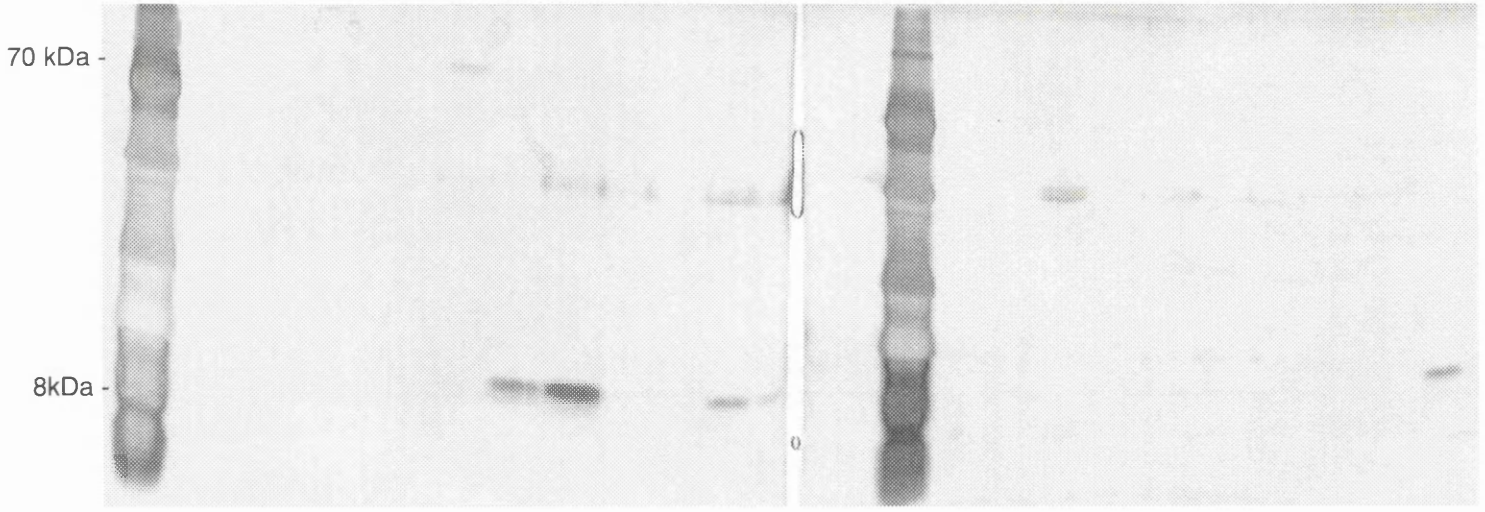
Analysis of the GI MIP-1α reverse phase column fractions on the KGM assay.



All the fractions eluted from the reverse phase column during the purification of GI H2 were analysed on the KGM assay. The fractions in water/acetonitrile/TFA were dried and then dissolved into PBS. Each fraction was assayed at a 5% dilution, ie 300μls of fraction in PBS. The controls C were 300μls of PBS. The most active fractions were fractions 19, 20 and 21.

Figure 25.

Silver stain of the reverse phase column purified fractions 15 to 31.

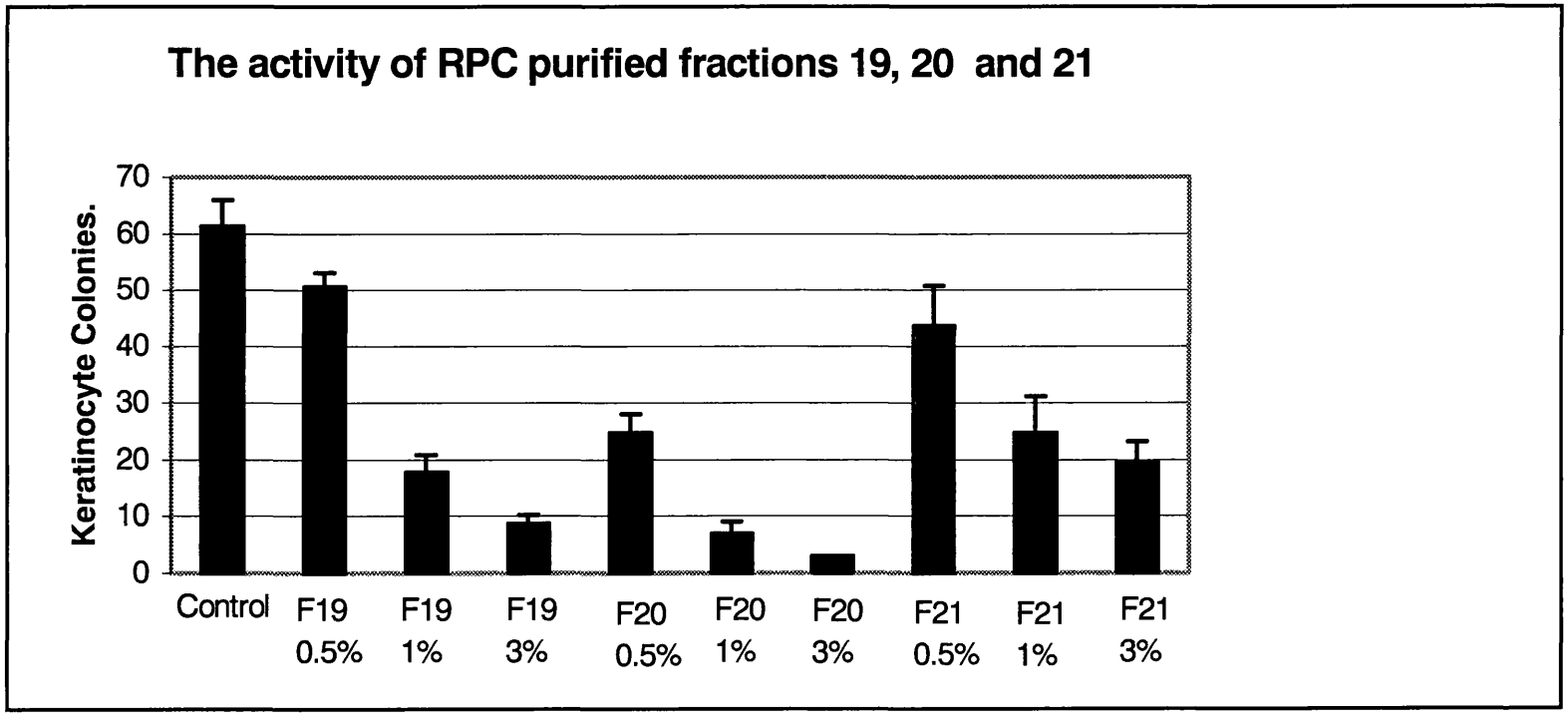


MWM 15 16 17 18 19 20 21 22 23 C MWM 24 25 26 27 28 29 30 31 C
(Numbers represent respective fractions).

C (control) represents 50ng of bacterial recombinant mu MIP-1a.
MWM represents the molecular weight markers.
This silver stained SDS-PAGE indicates that the majority of the MIP-1α is present in fractions 20 and 21.

Figure 26

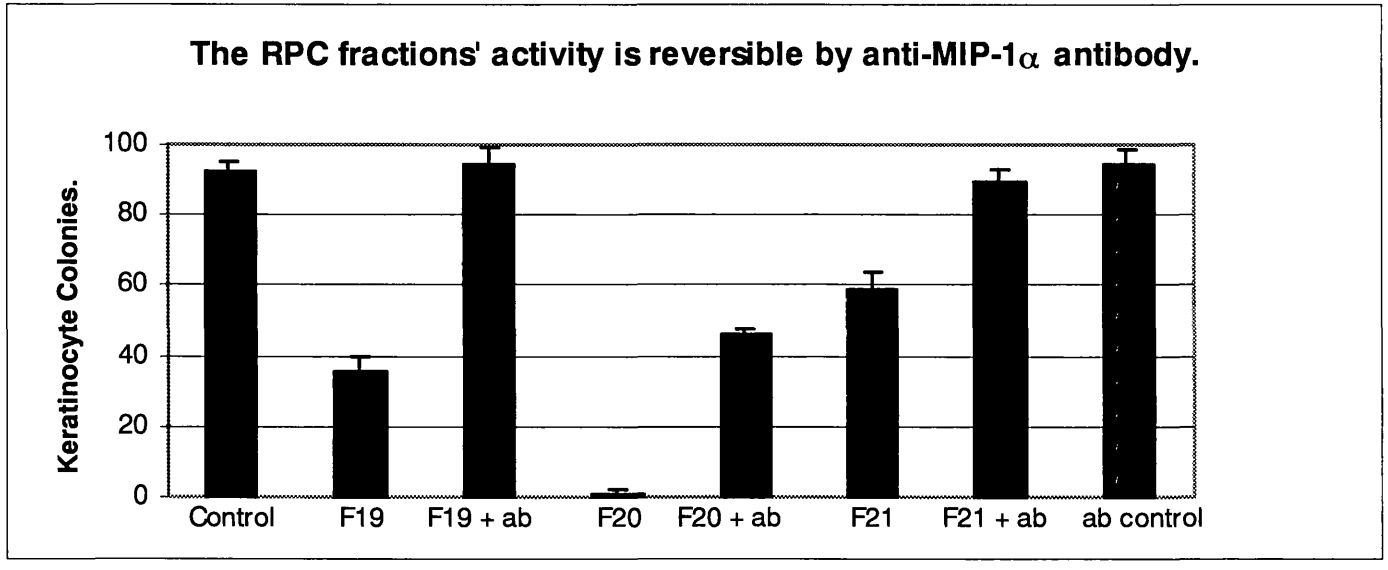
The activities of the reverse phase purified fractions 19, 20 and 21.



The active reverse phase purified fractions 19, 20 and 21 were assayed at the above dilutions. Fraction 20 appears to be the most potent, while fractions 21 appears to be the least potent.

Figure 27.

The activities of fractions 19, 20 and 21 are reversible by a specific polyclonal antibody to MIP-1α.

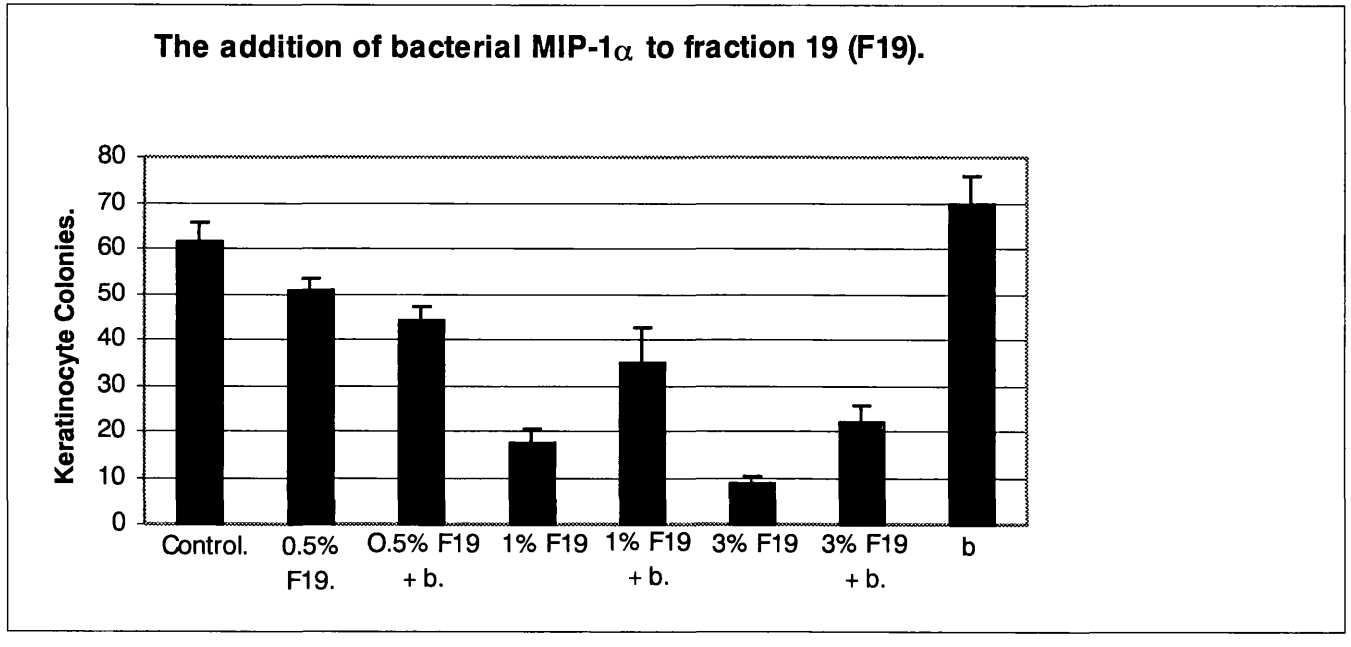


Control	300 μls of PBS.
F19	Fraction 19 3%.
F19 + ab	Fraction 19 3% + ab.
F20	Fraction 20 3%
F20 + ab	Fraction 20 3% + ab.
F21	Fraction 21 3%
F21 + ab	Fraction 21 3% + ab.
ab Control	Antibody control (100μls of 1mg/ml).

Quantity of antibody used: 100 μs of 1mg/ml (R&D systems).

Figure 28.

The activity of fraction 19 is not enhanced by the addition of bacterial recombinant MIP-1α.



The activity of fraction 19 was not potentiated by the addition of 100μls of 10μg/ml of bacterial recombinant MIP-1α.
On the contrary the above results suggest that the bacterial recombinant protein decreases the potency of the active fraction.
The results obtained above for the 0.5 % dilutions of F19 and bMIP-1α was not statistically significant.
The results obtained with the 1% dilution of F19 may be significant (p = 0.049).
bMIP-1α appears to significantly alter the inhibitory activity of the 3% dilution of F19 (p = 0.013).

3.7. Amino-terminally truncated MIP-1α mutants.

Amino-terminally truncated MIP-1α mutant cDNAs were generated by overlap extension PCR and inserted into the plasmid pMTx (as described in methods section 2.13.5.). These mutants were initially synthesised in COS 7 cells. The obvious disadvantage of pursuing this course of action is that the COS 7 cell system appears to generate a heterogeneous mixture of MIP-1α variants. However our studies suggested that the COS 7 cell transfection system primarily generates the protein encoded by the transfected cDNA. Therefore if amino-terminal truncation does result in a protein that is considerably more potent than the wildtype molecule (perhaps 100-1000 fold more active) we postulated that there should be very significant differences in the activities of the COS 7 cell derived truncated mutants despite the heterogeneity of the secreted chemokine.

Since previous studies have shown that the amino acids next to the conserved cysteines in a number of chemokines are crucial to receptor binding it was decided to generate a number of amino terminally truncated mutants extending to the alanine at position 10 next to the two cysteines.

Amino-terminally truncated MIP-1α mutants.

APYGADTPTACC.....	wildtype
PYGADTPTACC.....	-1
YGADTPTACC.....	-2
GADTPTACC.....	-3
ADTPTACC.....	-4
DTPTACC.....	-5
TPTACC.....	-6
PTACC.....	-7
TACC.....	-8
ACC.....	-9

As described in the methods section the -1 and -7 mutants were not secreted at detectable levels. This was not entirely surprising since the amino-acid proline at position +1 is known to inhibit signal peptidase activity, therefore preventing the secretion of these chemokines (see section 2.13.5). In view of this, the proline at position +1 in these two mutants was replaced by an alanine to generate a -1 P→A and a -7 P→A mutants.

The -1 and -7 proline to alanine (P→A) mutants.

APYGADTPTACC... Wildtype.

AYGADTPTACC... -1 P→A

ATACC... -7 P→A

These alanine to proline mutants were both secreted by the COS 7 cell expression system. However further analysis revealed that most of these mutants were secreted at considerably lower concentrations than the wildtype MIP-1α protein. Indeed the -5 and the -9 mutants appeared to be poorly secreted (see figure 29). This made quantitation of the mutants to equivalent concentrations for bioassay difficult. Furthermore since these mutants were poorly secreted, the purification and attempted concentration of these proteins resulted in an increase in the concentration of the background proteins. When the MIP-1α mutants were equilibrated to roughly equivalent concentrations very variable concentrations of these background contaminant proteins were obtained. This was a significant concern since the small quantities of MIP-1α mutants generated prevented further protein purification. However limited analyses of these mutants in both the KGM assay and in the CFU-A assay were performed. The results, presented in figure 30, appeared to suggest that amino-terminal truncation did not generate a potent kerato-inhibitory MIP-1α species. Amino-terminal truncation did however alter the activity of MIP-1α in the CFU-A assay (figure 31). These results suggested that the sequential amino-terminal truncation of the first four amino acids does not significantly alter the activity of MIP-1α on the CFU-A assay. However further truncation beyond the -4 amino-acid results in significant loss of activity, with complete loss of activity after the -6 truncation.

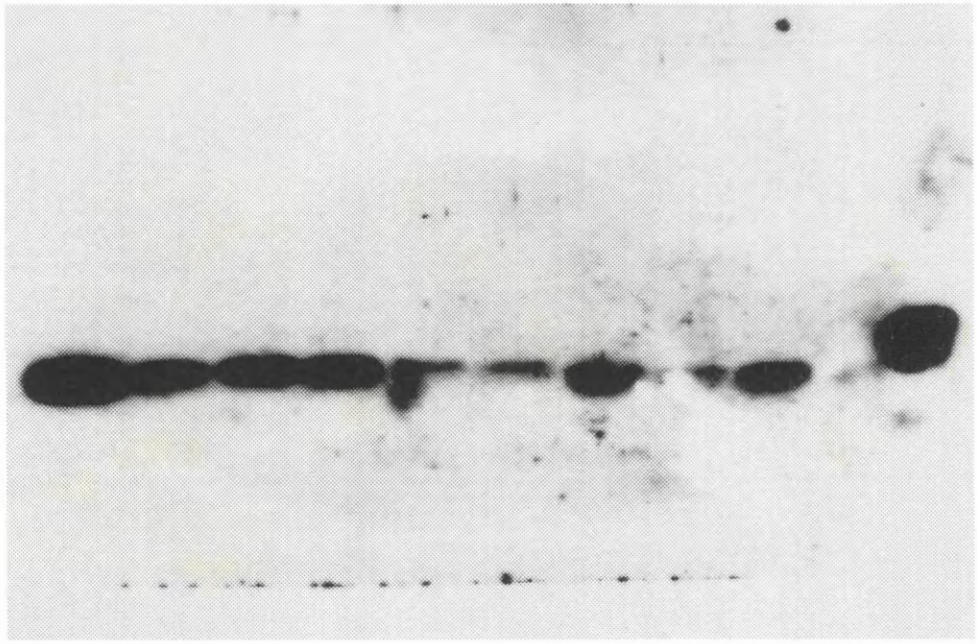
The results obtained with the COS 7 cell derived proteins seemed to unequivocally show that MIP-1α does not become a potent keratinocyte inhibitory protein when truncated at the amino-terminus by between 1 and 9 amino-acids. Electrospray mass spectrometry analyses had however suggested that COS cell transfection may generate a complex mixture of MIP-1α variants. It was therefore possible that this had modified the result of the COS 7 derived mutants assays. The inability to generate larger quantities of purified mutants for analysis, by Edman degradation protein sequencing and electrospray mass spectroscopy, meant that it had to be assumed that the mutant MIP-1α cDNA constructs were mainly secreting the expected truncated protein variants. Furthermore the low concentrations of

MIP-1α mutants generated, and the presence of significant concentrations of contaminating proteins in these preparations, may have masked the anticipated activity.

In view of this, human MIP-1α amino-terminally truncated mutants were generated in the *Saccharomyces cervisae* strain MC2 (Clements et al 1992) in collaboration with British Biotechnology (BBT: Oxford, UK). These mutant proteins were made as a fusion to pre-pro yeast alpha-factor. The yeast cultures yielded the MIP-1α mutants in the culture medium, following which these proteins were then purified by reverse phase HPLC by BBT. Initial studies with these proteins suggested in a number of assays that the -6 amino-terminally truncated form was significantly active in the inhibition of keratinocyte proliferation in the KGM assay with an ED50 of between 10-30ng/ml (Figures 32,33). Further analyses however detected significant variability in the keratinocyte inhibitory activity of this mutant and we have been unable to consistently observe this documented inhibition in all our assays. This inconsistency had been previously observed with the -4 human non-aggregating MIP-1α mutant and its cause has not as yet been defined. It may however be due to changes in the protein's three dimensional structure induced by, for example, repeat freeze-thawing.

Figure 29.

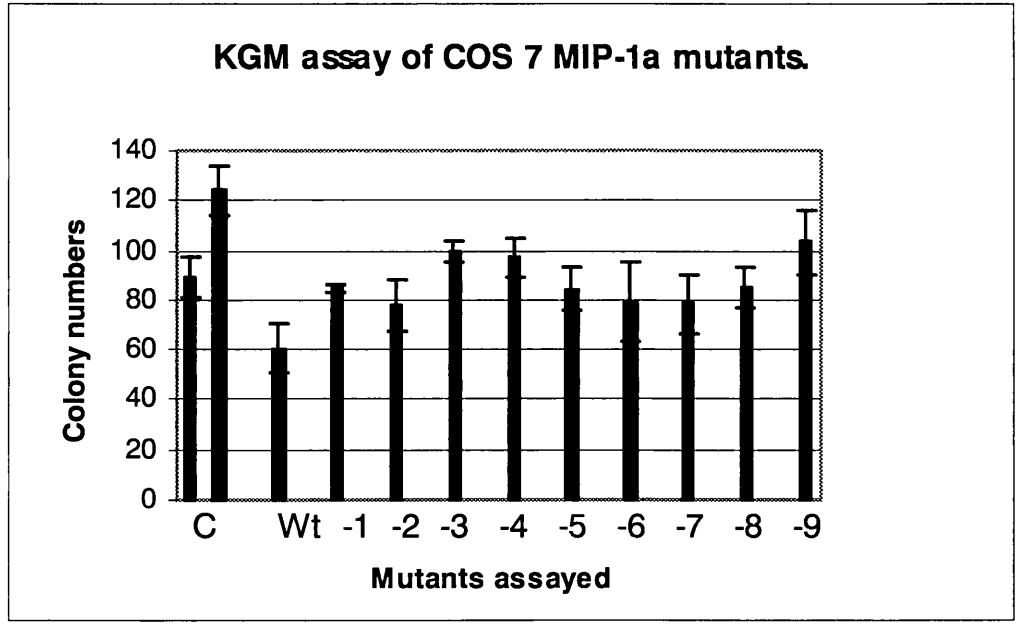
Western blot of the MIP-1α mutants.



Wt	-1P to A	-2	-3	-4	-5	-6	-7P to A	-8	-9	Bacterial

The skewed electrophoretic mobility of the bacterial recombinant MIP-1α in this 15% SDS-PAGE gel is artefactual.
The wildtype COS 7 cell protein has a very similar electrophoretic ability to the truncated mutants in a 15% SDS-PAGE gel.
The -4, -5 and -9 mutants were not secreted very well in this series of COS 7 cell transfections.
Control transfections with no plasmid were also carried out and this supernatant was used as a control in the mutants KGM assays.

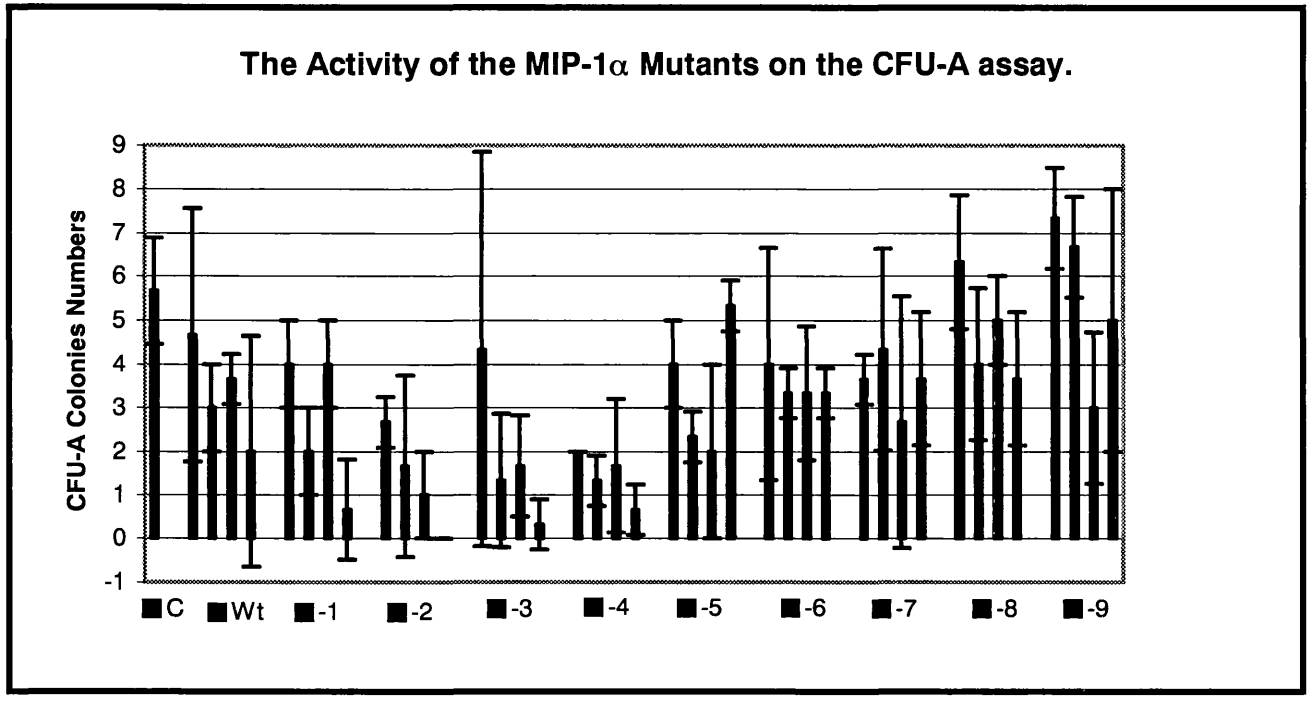
Figure 30.
KGM assay of COS 7 murine MIP-1α mutants.



- The COS 7 MIP-1α mutants were equilibrated by densitometry.
- The mutants were assayed at 1% dilutions (~1ng/ml).

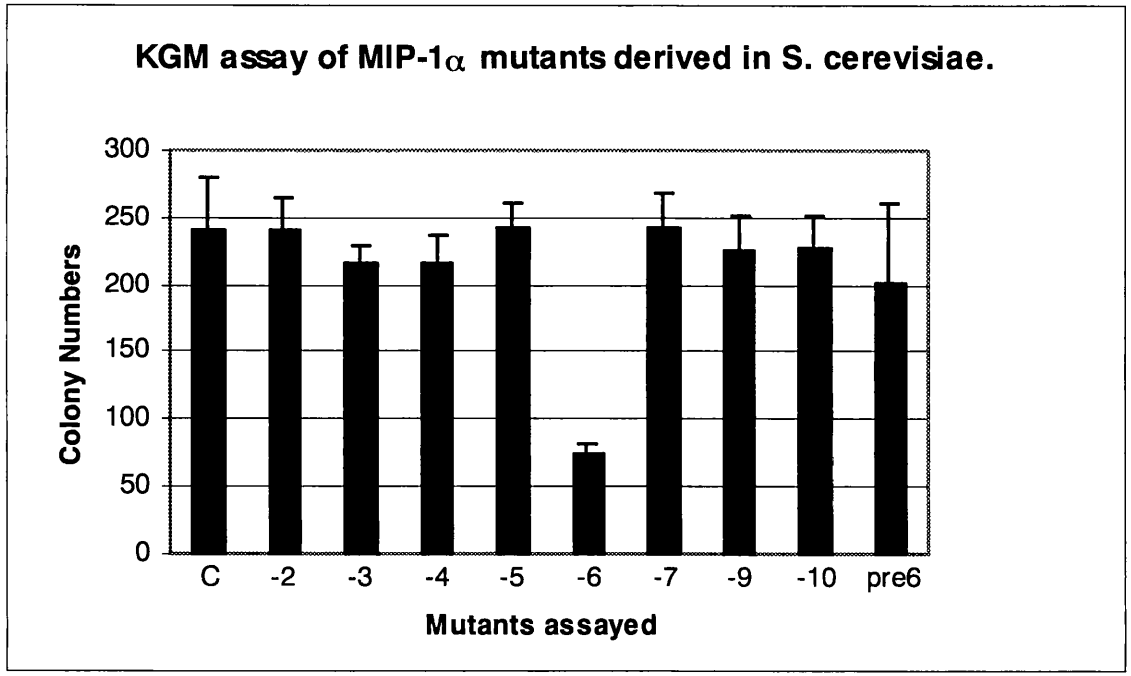
Figure 31.

CFU-A assays of amino-terminally truncated mutants.



- The amino-terminally truncated (COS 7 cell derived) murine MIP-1α mutants were bioassayed on the CFU-A assay. The mutants were all normalised to 1μg/ml. 10μls, 25 μls , 50 μls , 100 μls of each mutant preparation were added to these assays.

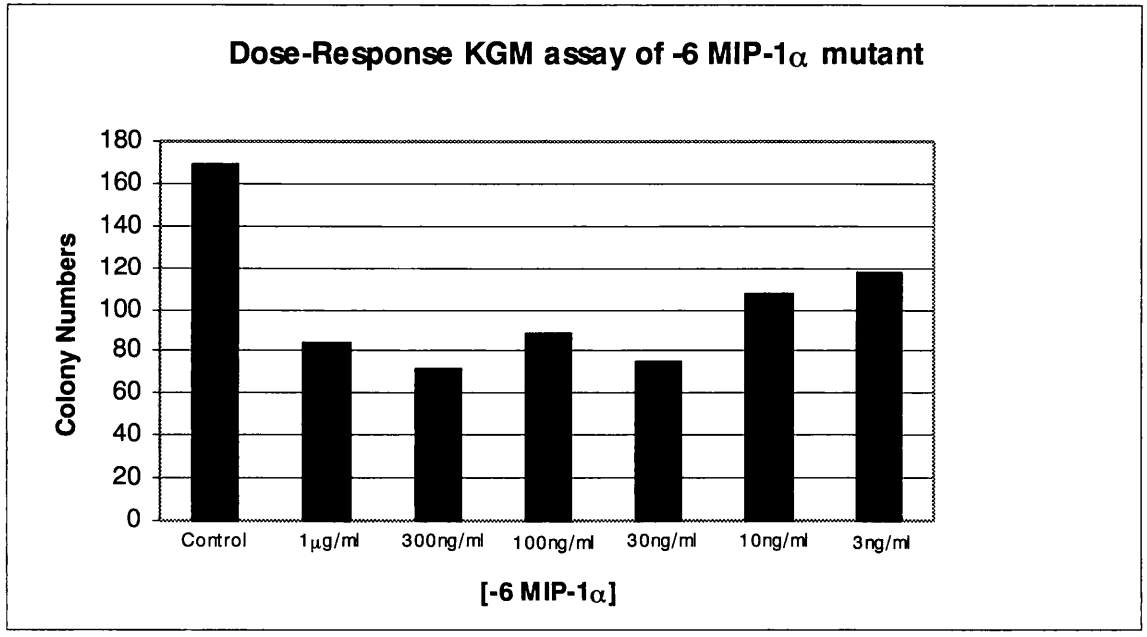
Figure 32.
KGM assays of MIP-1α mutants derived in Saccharomyces *cervisiae*.



- The mutants were assayed at 1% dilutions (~10ng/ml).
- Pre 6 (the - 6 mutant prior to fusion protein cleavage) was assayed as a control for the -6 mutant.

Figure 33

Dose-Response Activity of the -6 mutant in the KGM assay.



- This assay suggested that the -6 mutant was able to inhibit keratinocyte proliferation in a dose-response dependent manner with an ED50 of ~30-100ng/ml.

4. Discussion.

A number of studies have shown that chemokines often display diverse functions. It is clear that the chemokine MIP-1α is not only a potent inflammatory mediator but also an inhibitor of primitive haemopoietic cell proliferation (section 1.3.3.7.). This inhibitory activity of MIP-1α on primitive haemopoietic cells led us to study the activity of MIP-1α on the proliferation of other cell types. Initial investigations showed that a partially purified form of murine MIP-1α, derived from COS 7 cells, inhibited the proliferation of clonogenic human epidermal keratinocytes *in vitro* in the serum-free KGM assay. Furthermore this activity is reversible by specific polyclonal antibodies to bacterial recombinant murine MIP-1α, suggesting that the keratinocyte inhibitory activity of this partially pure preparation is due to the chemokine. The pure bacterial recombinant MIP-1α protein does not however inhibit keratinocyte proliferation although in common with COS MIP-1α it is active in inhibiting haemopoietic stem/progenitor cell proliferation in the CFU-A assay (Section 3.1). The experiments described have attempted to investigate the mechanism through which MIP-1α is activated to inhibit keratinocyte proliferation.

4.1. The Activity of Heparin Purified COS MIP-1α.

These experiments have shown that although the active COS MIP-1α is a very pure preparation (90-95% pure) it does contain a small number of contaminants (Section 3.1.1.). The initial concern was that the kerato-inhibitory activity could have been due to very low concentrations of some other inhibitory molecule such as TGF-β. However TGF-β1 was not detectable by ELISA in the active, blue sepharose purified, COS MIP-1α (section 3.3.1.). Furthermore specific polyclonal anti-MIP-1α antibodies were able to reverse the COS MIP-1α activity. This commercially available antibody preparation to bacterial MIP-1α was unlikely to be able to neutralise the activity of any other inhibitory molecule in COS MIP-1α (section 3.1.4) . However in order to try and exclude the presence of TGF-β2 and TGF-β3 the Latency Associated Peptide LAP was assayed in the presence of COS MIP-1α. These results were confounded by the significant increase in keratinocyte proliferation induced by LAP presumably through the inhibition of autocrine TGF-β making these KGM assays difficult to

interpret (section 3.3.4). Nevertheless since the anti-MIP-1α antibody did not alter colony formation in the control plates it appears that this antibody was not capable of interfering with the activity of TGF-β. These studies therefore suggested that the keratinocyte inhibitory activity of COS MIP-1α is unlikely to be due to TGF-β or any other contaminant, and suggested that MIP-1α is an inhibitor of clonogenic keratinocyte proliferation.

4.2. The Keratoinhibitory Activity of COS MIP-1α: Co-Factor or Structural Activation?

Since bacterial recombinant MIP-1α was inactive in the KGM assay, we postulated that the activity of COS MIP-1α was due to either

I) The presence of an activating co-factor, or

II) Some form of structural alteration not detected by SDS-PAGE analyses.

The chemokines are known to bind heparin and a number of other proteoglycans. These polysulphated molecules have been previously shown to enhance the neutrophil chemotactic activity of the CXC chemokine IL-8 (Webb et al 1993). We therefore postulated that COS 7 cell derived proteoglycans may be able to activate the kerato-inhibitory activity of MIP-1α. However heparin, heparan sulphate and chondroitin sulphate did not activate MIP-1a (Section 3.2.1). Moreover our studies were unable to detect any evidence to suggest the presence of a COS 7 cell derived activating co-factor (Section 3.2.2). COS 7 cells did not appear to constitutively secrete a MIP-1α co-factor. Furthermore COS 7 cells did not secrete a co-factor in the presence of the chemokine confirming that MIP-1α does not induce an activating co-factor. Interestingly however our results suggest that the bacterial recombinant form of MIP-1α can weakly antagonise the activity of the COS MIP-1α preparation (Sections 3: *figure 28*). This was seen both when heparin purified COS MIP-1α (figure 9) and when reverse phase purified COS MIP-1α (figure 28) were incubated with bacterial MIP-1α. The results were however statistically significant only in the latter experiment. This discrepancy is probably related to the low level of keratinocyte inhibition seen in figure 9 with 0.05% GI MIP-1α. When more significant inhibition is generated (figure 28), the antagonism becomes statistically significant. The results from both sets of experiments overall suggest that

bacterial MIP-1α weakly antagonises the activity of COS MIP-1α. This antagonism could be due to either protein aggregation or direct competition for the keratinocyte MIP-1α receptor. As discussed in Section 1.3.3.3, MIP-1α has a tendency to aggregate at higher concentrations. This aggregation may result in the sequestration of the kerato-inhibitory form of the chemokine by bacterial MIP-1α, reducing the binding of the active molecule to the receptor. However the non-aggregating murine MIP-1α mutant M1, which is only able to form tetrameric aggregates, was still able to act as a weak antagonist. This suggested that the antagonistic effect of bacterial MIP-1α was a result of direct although weak competition for the keratinocyte MIP-1α receptor. These studies therefore overall suggested that the keratinocyte inhibitory activity of MIP-1α does not require a co-factor and that the bacterial protein may still be able to bind the receptor and antagonise the activity of COS MIP-1α.

4.3. Purified COS MIP-1α is still active.

We were therefore unable to demonstrate the presence of an activating MIP-1α accessory factor in COS MIP-1α (Section 3.2.). Further evidence against this hypothesis came from the analysis of the activity of Blue Sepharose purified COS MIP-1α (Section 3.3.). Previous work in this laboratory had shown that MIP-1α binds very avidly to Blue Sepharose and can only be eluted from a Blue Sepharose column with high concentrations of $MgCl_2$. Blue Sepharose affinity chromatography was therefore used to further purify COS MIP-1α. This resulted in a very pure preparation of the chemokine (figure 10). In fact careful analyses of radiolabelled B2 revealed a single 8kDa MIP-1α band with no evidence of any contaminating proteins despite prolonged exposures to enhance the detection of background proteins (figure 11).

B2 was still active in the KGM assay and this activity was reversible by anti-MIP-1α antibodies (Section 3.3.2). This implied that the keratinocyte inhibitory activity of COS MIP-1α was unlikely to be dependent on the presence of a co-factor, but that some form of structural alteration brought about by COS 7 cells was activating the chemokine. This inhibitory activity of COS MIP-1α also appeared to be quite sensitive to inactivation by the high concentrations of the cation Mg^{++} used in these purification experiments (Section 3.3.3.). Mg^{++} is recognised to be the most effective cation in the Hofmeister ion-protein

interaction series and may be inactivating COS MIP-1α by altering the protein's three-dimensional structure.

4.4. The Structural Activation of MIP-1α.

Our results strongly suggested that MIP-1α is activated by some form of structural alteration to become an inhibitor of keratinocyte proliferation. However analyses by glycine SDS-PAGE gel electrophoresis revealed no obvious differences between bacterial MIP-1α and COS MIP-1α. The activating process therefore probably results in either a very subtle alteration in the COS MIP-1α molecule's structure or a radical alteration in a small fraction of the COS 7 MIP-1α. A conformational change in the three-dimensional structure of the molecule brought about by the mammalian cell expression system but not by the bacterial expression system may explain these findings. On the other hand since MIP-1α is a relatively small protein, minor changes in the chemokine's primary structure by amino-terminal or carboxy-terminal truncation may not be easily detected by SDS-PAGE gel electrophoresis. However careful analyses utilising Tricine SDS-PAGE gel electrophoresis, which is very sensitive to minor changes in protein mass/charge, did not demonstrate any detectable differences between bacterial MIP-1α and COS MIP-1α. In order to investigate this further, the pure form of COS MIP-1α, B2, was analysed further by protein sequencing (Section 3. 5.1). Initial results with a number of different COS MIP-1α samples revealed only the full-length wildtype murine MIP-1α sequence (APYGADT.....), identical to the bacterial recombinant protein sequence. However the sequence ADT... was also detected at very low concentrations. The significance of this result was not clear, however it suggested the presence of a -4 amino-terminally truncated MIP-1α molecule. Since no other sequences were detected, and since there is a precedent for activation by amino-terminal truncation in the chemokine family, we postulated that this -4 amino-terminally truncated molecule may be the active species in COS MIP-1α (Section 3.5.1.)

4.5. The Amino-Terminus in the Chemokine Family of Proteins.

Sequence analysis of chemokines has previously revealed that a number of these proteins can occur in multiple forms that differ through truncation at the amino-terminus (Section 1.3.3.4). IL-8, the prototypic CXC chemokine, has been previously shown to exist in at least 6 different amino-terminally processed forms (Van Damme 1991). Different amino-terminally truncated forms of natural IL-8 have been observed in a number of laboratories (Section 1.3.1.4.). Furthermore it appears that the composition of IL-8 depends on the cell source, rather than on the purification method (Van Damme 1989; Schroder 1990b). Since the amino-terminus of this molecule has been shown to be important for receptor binding, it is clear that this amino-terminal processing is important for the biological activity of the molecule. Similarly the CXC chemokine Platelet Basic Protein (PBP) has also been shown to be processed to generate several amino-terminal truncated forms with quite different bioactivities. The parent molecule PBP is in fact converted by exposure to various proteases to a number of different molecules with different activities: CTAP-III, β-Thromboglobulin, NAP-2 (Walz et al 1989; Walz and Baggiolini 1990). Platelet factor 4 (PF-4) is another CXC chemokine that has been shown to naturally exist in an amino-terminally truncated state. A 70 amino-acid protein, the parent full-length molecule has been shown to be able to inhibit endothelial cell proliferation by blocking cell-cycle progression through S-phase at unusually high concentrations (micromolar range). Other studies have shown that when isolated from activated human leukocyte culture supernatants, PF-4 is a significantly more potent inhibitor of endothelial cell prolieration (>50 fold more potent). This enhanced activity is due to an amino-terminally processed form of PF-4 generated by peptide bond cleavage between Thr-16 and Ser-17, a site which is surprisingly located downstream from the highly conserved and structurally important CXC motif (Gupta et al 1994 and 1995). This N-terminal processing of PF-4 appears to represent an important mechanism for regulating the activity of PF-4 (Section 1.3.1.1.).

The amino-terminus of the C-C sub-family of chemokines has also been shown to be critically important in receptor binding (see chapter 1.3.3.4). Studies on the C-C chemokine MCP-1 have shown that minor alterations in the amino-terminus of the parental molecule can result in significant changes in the bioactivity of this protein. Removal of one amino-acid from MCP-1 to generate a 2-76 form significantly alters the activity of this molecule. While the

wildtype molecule is a potent basophil activator with no effect on eosinophils, the 2-76 molecule is a potent eosinophil chemoattractant with little effect on basophils (Weber et al 1996). Extension of RANTES by one amino-acid (a methionine) at the amino-terminus results in a RANTES analog that is not only inactive, but also functions as a potent antagonist of the wildtype molecule (Proudfoot et al 1996). Furthermore a derivative that was created by chemical modification of the RANTES amino-terminus, aminooxypentane (AOP)-RANTES, is a subnanomolar antagonist of CCR5 (Simmons et al 1997; Mack et al 1998). It is therefore clear that the amino-terminus helps define the biological activity of the chemokines.

It has also been suggested that proteolytic modification of the chemokines by amino-terminal truncation may be an important physiological mechanism for modulating and determining the biological activity and receptor binding of chemokines. This has been confirmed by recent studies which have shown that a truncated form of the parental RANTES molecule, -2 RANTES (3-68) is secreted by dermal fibroblasts (Noso et al 1996). RANTES has also been shown to be a substrate for the leukocyte activation marker CD26 (Oravecz et al 1997). CD26 possesses dipeptidyl peptidase IV activity and converts the full-length Rantes molecule to the -2 amino-terminally truncated Rantes (3-68). This truncation significantly alters the function of the Rantes molecule. There are therefore precedents for functional modulation of the chemokines by amino-terminal truncation. Activation of MIP-1α through this mechanism would therefore appear to be an attractive means for generating the keratinocyte inhibitory activity in COS MIP-1α.

4.6. MIP-1α, Signal Peptidase and Amino-terminal truncation.

Most of our experiments in the laboratory involved using the murine COS MIP-1α protein on human epidermal keratinocytes. Human MIP-1α, also known as LD78 is about 75% homologous to murine MIP-1α. It was first cloned from tonsillar lymphocytes by Obaru et al in 1986. This sequence suggested that the LD 78 signal peptidase cleavage site resulted in a -4 form of MIP-1α. However more recent studies have suggested a different cleavage site. Signal peptidase cleaves the transient secretory signal sequence, found on most secretory proteins, from the mature protein. This sequence serves to initiate protein export across the membrane of the endoplasmic reticulum. A number of studies have shown that the cleavage site for this enzyme is defined by the signal sequence which comprises three distinct regions:

a basic N-terminal region, a central hydrophobic region and a more polar C-terminal region (Von Heijne 1986). The cleavage site is usually 4-6 amino-acids downstream from the end of the hydrophobic region with residues occupying positions -1 and -3 relative to the cleavage site being the most important ones in defining the precise position of cleavage. This observation has been formally incorporated into the "-1, -3 rule" by Von Heijne (1986). This rule has been used very successfully to predict the most likely site of signal peptidase cleavage. We have been advised that murine MIP-1α does in fact have two potential signal peptide cleavage sites separated by 4 amino acids (personal communication Von Heijne 1995). Therefore signal peptidase cleavage may result in a -4 amino-terminally truncated murine MIP-1α which may be the kerato-inhibitory species. Previous studies have however predominantly identified the full-length protein suggesting that, if this is the mechanism of activation, this is the preferred cleavage site. Interestingly however other studies have also identified a -4 human MIP-1α variant. Cocchi et al (1995) characterised a CD8+ T cell-line derived HIV suppressive factor as -4 amino-terminally truncated human MIP-1α. The mechanism which generates these two different amino-termini has not been identified. A number of different possibilities exist:

I. Different genes (ie LD78α and LD78β) expressing proteins with different amino-termini.

II. Alternative mRNA splicing sites.

III. Alternative signal peptidase cleavage sites.

IV. Post-translational modification intracellularly or extracellularly.

I. Different genes (LD78α and LD78β).

There are three different although highly homologous human MIP-1α genes (Nakao et al 1990), termed LD78α, LD78β and LD78γ. LD78γ is a pseudo-gene and is not expressed. However LD78α and LD78β both appear to be expressed in a number of cell lines (Nakao et al 1990). These two proteins are almost identical but recent studies in our laboratory suggest that they may be structurally and functionally distinct (Nibbs and Graham: personal communication). They differ only in five amino-acids: two of these changes are conservative serine to glycine substitutions at the carboxy-terminus, while the other three changes are non-conservative and are clustered around the signal peptide cleavage site. Nakao *et al*

(1990) suggested that the signal peptide cleavage sites of these two human MIP-1α proteins are at the same position. It is likely however that due to the substrate specificity of eukaryotic signal peptidase, according to Von Heijne's method (Von Heijne 1986) for predicting signal sequence cleavage sites, that these two highly homologous genes result in proteins with different amino-terminal lengths. The mature LD78β secreted protein should therefore be secreted as a full-length protein similar in length to the murine protein (see figure overleaf), while the mature LD78α may be secreted as a -4 truncated molecule (when compared with the murine protein).

The MIP-1α (LD78) Signal Sequences and Putative Signal Peptidase Cleavage Sites.

Murine MIP-1α

MKVSTTALAVLLCTMTLCNQVFS↓APYGADTPTACC.....

Human LD78α

MQVSTAALAVLLCTMALCNQF* S ASLA↓ADTPTACC.....

Human LD78β

MQVSTAALAVLLCTMALCNQVLS↓APLAADTPTACC.....

The putative cleavage sites of signal sequences are indicated by arrows. An asterisk is inserted to maximise the homology. The amino-acid differences between LD78α and LD78β are underlined.

II. Alternative mRNA Splicing Sites.

A novel β-chemokine CKβ8 (also known as Myeloid Progenitor Inhibitory Factor-1: MPIF-1), which exhibits 51% identity and 67% similarity to human MIP-1α has recently been characterised (Patel et al 1997). More recent studies have in fact identified two expressed splice variants of this β-chemokine termed CKβ8 and CKβ8-1 (Youn et al 1998). CKβ8 mRNA was found to be 51 nucleotides (17 amino-acids) shorter than CKβ8-1. Both these molecules were found to be agonists of CCR1. This was the first example of alternative splicing producing two active β-chemokines from a single gene. This mechanism could be employed by other β-chemokine genes, however an alternatively spliced form of MIP-1α

mRNA has never been cloned despite extensive PCR based studies. This makes alternative MIP-1α mRNA splicing very unlikely.

III. Alternative Signal Peptidase Cleavage Sites.

This has already been discussed and could potentially explain the presence of the full-length and the -4 truncated protein in COS 7 MIP-1α. However we are not aware of a precedent for this since signal peptidase only appears to cleave at a single site in any one protein.

IV. Post-Translational Modification Intracellularly or Extracellularly.

Several examples of post-translational modification of both α– and β- chemokines have been recorded. The amino-terminus and function of the β-chemokine Rantes has been shown to be altered *in vivo* by the dipeptidyl-peptidase IV activity of CD26 which generates -2 (3-68) Rantes from the full-length molecule (Oravecz et al 1997). CD26 is expressed on the cell surface of macrophages and T lymphocytes where it presumably functions. It specifically recognises the N-terminal motif NH_2-X-Pro (where X is any amino-acid). Since several chemokines, including muMIP-1α have this amino-terminal motif it is likely that CD26 may regulate the function of a number of chemokines. Other studies have recently shown that another β-chemokine, the 76 amino-acid MCP-1, is secreted by mononuclear cells as a combination of at least three different processed forms: MCP-1 (5-76), MCP-1(6-76) and a carboxy-terminally processed form MCP-1(1-69) as well as the wildtype glycosylated and non-glycosylated forms (Proost et al 1998). MCP-2 was also found to be secreted as a mixture of amino- and carboxy-terminally processed forms: MCP-2(6-76), MCP-2(1-74). Interestingly these molecules displayed significantly different activities. It is therefore clear that post-translational processing of the chemokines adds another level of complexity to what is already an intricate system comprising many molecules with overlapping functions. However it would appear that post-translational modification at the amino- and carboxy-termini is used to modulate the activities of these molecules.

Our findings had suggested that COS 7 cells secrete both the full-length and a truncated form of MIP-1α. We therefore postulated that MIP-1α is activated to become an inhibitor of keratinocyte proliferation in the KGM assay by the amino-terminal truncation of 4 amino-

acids. However the mechanism of activation both *in vitro*, in the COS 7 cell transfection, and *in vivo*, possibly in the epidermal Langerhans cell, could be due to any one of the above.

4.7. Amino-terminally truncated MIP-1α and keratinocyte proliferation .

As alluded to above there has been some confusion in the literature regarding the mature amino-terminus of huMIP-1α (Section 3.5.1). In a series of reviews on the chemokine family a number of different mature amino-terminal human MIP-1α sequences have been listed. Schall (1991) suggested that the amino-terminus of human MIP-1α was SLAADTPACC...., while Stoeckle et al (1990) suggested that the amino-terminus was ADTPTACC...., as indicated by Obaru et al (1986) who first identified the gene. Miller and Krangel indicate that although the signal peptide cleavage site for murine MIP-1α was known, the signal peptidase cleavage site for human MIP-1α had not been defined. Other studies have suggested that signal peptidase cleavage results in a molecule with an amino-terminus that is similar to the murine amino-terminus: ASLAADTPTACC...(Nakao et al 1990). It appears however that none of these investigators have sequenced the amino-terminus of human MIP-1α.

Previous experiments carried in this laboratory investigating the aggregation of MIP-1α had involved the generation of non-aggregating bacterial murine and human recombinant MIP-1α peptides in collaboration with R&D systems (Minneapolis). The murine non-aggregating MIP-1α mutants were generated as full-length molecules (Graham et al 1994). The -4 amino-terminally truncated sequence for human MIP-1α described by Obaru et al (1986) was however used to generate the human non-aggregating MIP-1α mutants. A -4 truncated non-aggregating form of human MIP-1α, with wildtype activity in the CFU-A assay, was therefore available in our laboratory. This MIP-1α variant huM1 did however have three carboxy-terminal mutated amino-acids to prevent the non-covalent protein aggregation:

.....Q**K**YVS**D**L**E**LSA (Wildtype COOH terminus) *was mutated to*

.....Q**Q**YVS**N**L**Q**LSA (-4 non-aggregating human mutant COOH terminus).

The neutralisation of these charged amino-acids partly abrogates protein aggregation (Parker et al submitted: 1998). This -4 non-aggregating mutant was found to be able to inhibit

keratinocyte proliferation in the KGM assay (section 3.5.2/ figure 18). This activity was reversible with anti-human MIP-1α antibodies (figure 19). Our studies also showed that the full-length wildtype and non-aggregating murine mutant MIP-1α proteins were not active in the KGM assay, although they inhibited cell proliferation in the CFU-A assay. This indicated that the activity of the human non-aggregating mutant was not related to the loss of aggregation. This result suggested that the loss of 4 amino-terminal amino-acids resulted in the generation of a kerato-inhibitory huMIP-1α.

Significant variability in the keratinocyte inhibitory activity of different batches of human MIP-1α mutant was however noted. The reason for this variability was not clear although similar problems have been previously encountered by other investigators (Broxmeyer et al 1994). Broxmeyer indicates that significant variability in bioactivity has been observed with MIP-1α obtained from different sources.This has also been seen with different MIP-1α batches obtained from the same source. It has been suggested that this may be largely due to protein aggregation. However our findings suggest that differences in the amino-terminus of these MIP-1α preparations may also be important.

The use of 0.1 - 0.5% bovine serum albumin as a carrier protein in our experiments did decrease this variability, but it did not abrogate it. This suggested that this variability in activity may have been partly due to protein loss, however this should have been controlled for in all these experiments. There may be other reasons for this variability, perhaps relating to protein modification by repeat freeze thawing. Also the incomplete removal of trifluoroacetic acid (TFA) from the protein preparation by freeze-drying, prior to the dissolving of MIP-1α in PBS, may also perhaps explain some of this variability since recent studies have shown that minor variations in pH and sodium chloride concentration over a range of physiological and near physiologic conditions can have dramatic effects on chemokine to receptor binding (Dairaghi et al 1997). Although the experiments described analysing the activity of different MIP-1α preparations were carefully controlled for, the pH and sodium chloride concentrations in these assays were not routinely tested. Minor fluctuations in pH or salt concentrations in these assays could therefore be partly responsible for some of the variability seen.

These results did nonetheless suggest however that a bacterial recombinant -4 truncated mutant of human MIP-1α could inhibit keratinocyte proliferation in the KGM assay. It was also clear that this molecule was not as potent as the presumed active species in COS MIP-1α.

Our estimates suggested that the active species in COS MIP-1α was probably between 100-1000 times more potent. Therefore even if the -4 variant was active, it was unlikely to be the only active species in COS MIP-1α. In order to elucidate this further COS MIP-1α was analysed by electrospray mass spectroscopy. Electrospray mass spectrometry is an accurate means of obtaining molecular mass information on a wide variety of molecules with picomolar to femtomolar sensitivity. It is considerably more sensitive at detecting proteins than SDS-PAGE gel electrophoresis and has been widely used in biochemical research to analyse a number of different types of modifications in protein structure (Siuzdak et al 1994). MIP-1α aggregation made analysis by electrospray difficult, but the addition of acetic acid and acetonitrile prior to the injection of the preparation into the mass spectrometer appeared to resolve this problem (Graham et al 1992). Electrospray mass spectrometry of this acidified preparation of COS 7 MIP-1α suggested that this preparation comprises a number of different truncated MIP-1α variants (Section 3.5.3/ figure 20). These studies suggested that COS 7 MIP-1α may contain small quantities of different truncated forms of MIP-1α, ranging from -1 to -7. Altogether these analyses appeared to suggest that the inhibition of keratinocyte proliferation by COS 7 cell MIP-1α may be due to the presence of a structurally altered, amino-terminally truncated form of MIP-1α. Since the -4 MIP-1α molecule was not as potent as the COS MIP-1α preparation, we therefore postulated the presence of an alternative, more potent, amino-terminally truncated MIP-1α species in COS MIP-1α. In order to investigate this further we systematically generated a series of amino-terminally truncated MIP-1α mutants. Since we had no laboratory data to suggest truncation beyond the structurally conserved -CC- cysteine residues, we generated nine sequentially truncated mutants ranging from -1 to -9 MIP-1α.

4.8. Systematic amino-terminal truncation of the MIP-1α molecule.

A series of amino-terminally truncated MIP-1α molecules were generated both in COS 7 cells and in S. *Cervisae* and bioassayed in the KGM assay. Considerable difficulty was experienced in generating adequate amounts of COS 7 murine MIP-1α mutants because many of these mutants were poorly expressed compared with the wildtype molecule (Section 3.7.). The studies performed with these mutants suggested that amino-terminal truncation did

not potentiate the ability of wildtype COS MIP-1α to inhibit keratinocyte proliferation (figure 30). It is possible however that some of these mutants are not generated as the intended truncation mutant. These studies were also hindered by the inability to analyse the COS 7 mutants by either sequencing or electrospray mass spectrometry. Further studies using purified S.*cervisiae* derived mutants generated in collaboration with British Biotechnology were therefore performed. These assays initially suggested that truncation at the amino-terminus altered the keratinocyte inhibitory property of human MIP-1α, with the -6 mutants being the most active truncated species, with an ED50 of ~30ng/ml (figure 32). However these results could not be consistently repeated. The cause of this variability in activity was again unclear, although inactivation by repeat freeze-thawing could cause this loss of activity. It is also possible that alteration of the MIP-1α primary structure by amino-terminal truncation alone is not sufficient to generate a kerato-inhibitory molecule. Further modifications in the MIP-1α secondary and/or tertiary structure may be necessary to generate a MIP-1α molecule with keratinocyte inhibitory properties.

The COS 7 amino-terminally truncated MIP-1α mutants were also analysed in the CFU-A assay (figure 31). Amino-terminal truncation up to and including -4 MIP-1α had little effect on the activity of MIP-1α in the CFU-A assay, but truncation beyond this resulted in the loss of CFU-A activity, suggesting that the amino-terminal four amino-acids are critical to the activity of MIP-1α in this assay. Similar results have been obtained with the S. *cervisiae* mutants (G.J. Graham: personal communication).

4.9. Potential Alternative Activating Mechanisms.

These results have been unable to clearly define the activating mechanism of the keratinocyte inhibitory activity in COS 7 MIP-1α. Although further studies may still define -6 MIP-1α as the active species, if none of the amino-terminal mutants investigated thus far are the kerato-inhibitory species, a number of other potential mechanisms of activation may need to be considered.

a) Amino-terminal truncation beyond the -9 amino-acid.

Amino-terminal truncation beyond the -9 position tested so far should result in a MIP-1α variant that can be differentiated from the full-length molecule by SDS-PAGE analysis. However these studies suggest that the active species is present in COS 7 MIP-1α in very low concentrations. On the other hand since our analyses were unable to demonstrate a MIP-1α variant with this degree of amino-terminal truncation, and since the two conserved cysteines at positions +10 and +11 are essential to give rise to the disulphide bonds that maintain the β-chemokine three dimensional structure, we did not analyse truncations beyond the threonine at position 9. There is however a precedent for functional activation by amino-terminal truncation beyond these two conserved cysteines. Previous studies isolated a PF-4 derivative that is generated by peptide bond cleavage between Thr-16 and Ser-17, a site located downstream from the highly conserved and structurally important CXC, from activated human leukocyte culture supernatants (Gupta et al 1995). This truncated form of PF-4 acts as a potent inhibitor of endothelial cell proliferation and is ~50 fold more potent on endothelial cells than PF-4. Therefore it remains possible that MIP-1α is activated by COS 7 cells by N-terminal truncation beyond the CC motif. However unless such a molecule is detected in COS 7 MIP-1α attempting to construct further N-terminal mutants is not envisaged.

b) Carboxy-terminal truncation.

The carboxy-terminus is considered to be less important for chemokine receptor binding than the amino-terminus. This may however be a reflection of the difficulty inherent in carboxy-terminal sequencing. Previous studies have shown that a number of both α– and β-chemokines are secreted as a mixture of amino- and carboxy- terminal truncated

variants. The α-chemokine NAP-2 has been shown to naturally exist as a -4 carboxy-terminal truncated variant while the β-chemokines MCP-1 and MCP-2 both have been shown to occur naturally in carboxy-terminally truncated forms (Proost et al 1998). The functional significance of these truncations is not fully understood. Furthermore the purification and characterisation of these variants from the wildtype molecule has been found to be very difficult by a number of investigators. Our studies have been unable to exclude the presence of a carboxy-terminal truncation activation. It is important to note that the masses for at least the first two (-1 and -2) MIP-1α mutants detected by electrospray mass spectroscopy could indicate the presence of -1 and -2 carboxy-terminal mutants (amino terminal sequence APYG....; carboxy-terminal sequence ....ELNA). Therefore carboxy-terminal processing could explain the activity of COS MIP-1α. In order to investigate this, carboxy-terminal sequencing of highly purified and concentrated active COS MIP-1α fractions needs to be carried out. At present carboxy-terminal sequencing is not easily accessible. However recent developments in this area and in the field of mass-spectrometry using tandem HPLC and electrospray mass spectrometry may in the near future make automated sequencing of picomolar quantities of protein feasible (Wilm et al 1996). Further analysis of COS MIP-1α using these technologies may become available in the near future.

c) **Alterations in the Three-Dimensional Structure of MIP-1α.**

The studies described have suggested that modification of the primary structure of MIP-1α may be required for keratinocyte inhibition. This form of modification may result in changes in the secondary and perhaps also the tertiary structure of this protein. This may in turn result in the unveiling of specific residues that are critical for binding to the keratinocyte receptor. Alternatively it is possible that there are differences in the three-dimensional structures of COS 7 and bacterial recombinant MIP-1α that cannot be detectable by SDS-PAGE electrophoresis. Such differences could be responsible for the functional alteration observed. COS 7 cells may be more able to bring about this type of modification than either E.*coli* or S.*cervisiae.* Further studies comparing the structures of bacterial MIP-1α and COS 7 MIP-1α may become necessary, however these studies may be difficult to perform in view of not only the heterogeneity of COS 7 MIP-1α, but also the

difficulties encountered in generating sufficient amounts of COS 7 MIP-1α for these studies.

4.10. Future Studies.

a) Identification of a Different Source of Keratinocyte Inhibitory MIP-1α.

These studies have confirmed that COS 7 MIP-1α is able to potently and reversibly inhibit clonogenic keratinocyte proliferation *in vitro*. The precise nature of the activating mechanism has not been fully characterised but is likely to be due to some form of structural modification. Future studies should attempt to identify other sources of kerato-inhibitory MIP-1α. A potential source is the Langerhans cell cell-line XS52 (Xu et al 1995). This cell-lines have been derived from the epidermis of newborn mice and resemble resident epidermal Langerhans cells. We have now acquired this growth-factor dependent and slow-growing cell line which has been shown to express MIP-1α mRNA and protein (A. Takashima: Personal communication). Further studies with this cell-line as a potential source of kerato-inhibitory MIP-1α may shed some light on the mechanism of MIP-1α activation.

b) Identification of a Keratinocyte Cell-Line Responsive to COS MIP-1α.

The use of primary clonogenic human epidermal keratinocytes in these experiments has both advantages and disadvantages. These cells are as close as one can get *in vitro* to the epidermal basal and suprabasal keratinocyte stem/progenitor cell (Kolodka et al 1998). The cells in this assay are heterogeneous with respect to proliferation potential. Further heterogeneity can be introduced to these assays by the use of cells at different stages of passage, although this can be controlled for. The identification of a keratinocyte cell-line that is responsive to COS 7 MIP-1α would significantly facilitate further *in vitro* studies. Studies on a number of keratinocyte cell-lines including Balb-Mk, TFKs and in house transformed cell-lines have suggested that the Balb-Mk cell line may be able to respond to COS MIP-1α (R. Nibbs: personal communication). The use of this cell-line may prove valuable in the future, particularly for both receptor and signalling studies.

c) Characterisation of the The MIP-1α Keratinocyte Receptor.

The results described have confirmed the findings of Parkinson et al (1993) who showed that COS 7 cell derived MIP-1α inhibits the proliferation of epidermal keratinocytes in vitro. Clonogenic epidermal keratinocytes therefore appear to possess at least one receptor for this chemokine. Members of the chemokine family have been shown to interact with their target cells using members of the G-protein-coupled heptahelical receptor family. A number of α- and β- chemokine receptors have been cloned. These receptors are mainly specific for their respective subfamilies, although they often have significant overlap in their ligand binding profiles. At least nine β-chemokine receptors have been described so far. These receptors show significant sequence homology at a number of sites. This has been successfully applied in this laboratory by Dr R.J. Nibbs to clone a number of chemokine receptors including CCR1, CCR3, CCR5 and the novel chemokine receptor D6 using a PCR based approach (Nibbs et al 1997a and b). No keratinocyte MIP-1α receptor has however been cloned to date using this approach. Reverse transcriptase PCR using clonogenic human epidermal keratinocyte mRNA and oligonucleotide primers derived from regions that show conservation of amino acid sequence has also been attempted. This resulted in the cloning of a murine IL-8 receptor but no β-chemokine receptor was cloned. This may indicate that there is significant heterogeneity between the oligonucleotide sequences used in this degenerate PCR cloning strategy and the corresponding sites on the putative clonogenic keratinocyte receptor. Future studies will be directed at attempting to clone these growth inhibitory receptors. Receptor binding studies using the keratinocyte inhibitory form of MIP-1α may help in the identification of these receptors. These studies have not been possible to date because of the lack of adequate amounts of purified COS 7 cell MIP-1α protein.

d) Characterisation of the MIP-1α Signalling Pathways.

The identification of the active MIP-1α keratinocyte inhibitory species would allow the investigation of the MIP-1α signalling pathways. It would be particularly interesting to compare the mode of action of TGF-β and MIP-1α. Studies on the activity of MIP-1α on the cyclins, cyclin dependent kinases and their inhibitors as well as pRb and p53 may

explain the growth-inhibitory activity of MIP-1α. Further work involving techniques such as differential display or subtractive hybridisation may also be worthwhile pursuing and potentially could result in the cloning of as yet uncharacterised target genes.

e) *In vivo* studies.

Studies on the expression of MIP-1α in both normal, hyperproliferative and wounded epidermis are planned using both immunocytochemistry and in situ hybridisation. It would be interesting to find out whether MIP-1α expression co-localises with Langerhans cell markers such as CD1a, and whether other bone-marrow derived epidermal cells such as γδ T-cells, which can be induced to express MIP-1α *in vitro* (Boismenu et al 1996), express MIP-1α in the epidermis. It would also be interesting to investigate whether MIP-1α expression is altered in hyperproliferative disorders such as psoriasis. Previous studies have however suggested that MIP-1α protein expression is very difficult to detect using immunocytochemistry. Recent investigations also suggest that MIP-1α may be involved in murine skin wound repair (Di Pietro et al 1998). The study of MIP-1α expression and function in this model is particularly relevant since MIP-1α appears to have the ability to act both as an inflammatory mediator and an inhibitor of keratinocyte proliferation.

f) MIP-1α and the Langerhans cell.

Previous studies have shown that the primary source of MIP-1α expression in the epidermis is the Langerhans cell. We have (in collaboration with D. Klatzmann and S Holmes) examined the epidermis of transgenic mice whose epidermal dendritic cell population has been partially ablated through the targetted expression of Herpes simplex thymidine kinase and the use of gancyclovir. The epidermis of these mice had a reduced epidermal dendritic cell population (Salomon et al 1994), but no overt epidermal hyperplasia. There was however evidence of significant keratinocyte apoptosis in the epidermis of these mice suggesting a significant bystander effect. This made evaluation of the Langerhans celll loss difficult (S. Holmes: personal communication).

More recent studies have suggested that TGF-β1 knockout mice appear to have a defect in normal murine Langerhans cell development (Borkowski et al 1996, 97), the epidermis

of these mice being depleted of Langerhans cells. However studies have also shown that the epidermis in these mice contains hyperproliferative keratinocytes due to TGF-β loss (Glick et al 1993,1994) making it impossible to comment on the effect of Langerhans cell MIP-1α loss on epidermal keratinocyte proliferation. The study of keratinocyte proliferation in MIP-1α knockout mice is therefore currently underway.

g) Transgenic Mice

The ability to study the *in vivo* function of specific molecules using transgenic mouse technology has become a valuable tool for the investigation of the intricate biological processes involved in the regulation of the complex cellular processes that ultimately lead to cellular differentiation, proliferation or apoptosis. Targetted gene expression to a specific tissue, or inactivation of the gene of interest by homologous recombination, are now routinely used in the study of important cellular proteins.

MIP-1α Knockout Mice.

The biological role of MIP-1α *in vivo* has been examined in mice in which the gene encoding MIP-1α has been disrupted (Cook et al 1995). Although homozygous MIP-1α mutant (-/-) mice have no overt haematopoietic abnormalities, these mice have significantly altered responses to viral pathogens suggesting that MIP-1α is an important mediator of virus-induced inflammation *in vivo*. Studies are currently underway in this laboratory to investigate the *in vivo* role of MIP-1α in murine epidermis by examining the skin of MIP-1α knockout (-/-) mice. Preliminary results have shown that a proportion of these MIP-1α knockout mice have hyperplastic epidermis (Holmes and Mackie: personal communication). This phenotype may depend on the genetic background of the mouse as has been previously observed with TGF-β knockout mice (Schull et al 1992; Dickson et al 1995).

If these studies do in fact show that MIP-1α knockout mice have hyperplastic epidermis, further studies will be carried out involving the transplantation of normal bone marrow to these mice. Previous studies have shown that Langerhans cells are derived from bone marrow stem cells, and reside in the epidermis for long periods (Section 1.4.2.3).

Treatment of the epidermis with radiotherapy does however result in epidermal Langerhans cell ablation, as well as bone marrow aplasia. The transplantation of normal bone marrow, after radiotherapy, to these mice will result in the repopulation of the epidermis with MIP-1α expressing Langerhans cells. This may be able to reverse the epidermal and would conclusively demonstrate the paracrine role of MIP-1α.

Further studies on the effect of the loss of MIP-1α on tumour initiation, promotion and progression are also currently being carried out on these MIP-1α knockout mice. This studies will investigate the effect of this mutation on epidermal carcinogenesis.

Targetted MIP-1α Overexpression to the Epidermis.

Targetted overexpression of the active MIP-1α species to the basal /suprabasal layers of the epidermis using specific keratin promoters is also planned. A keratin 14 construct previously used for the targetted over-expression of IL-6 and KGF in murine epidermis has been acquired from the laboratory of Dr E. Fuchs (Turksen et al 1992). However a better understanding of the activating mechanism needs to be acquired before these studies can be performed.

4.11. Potential Clinical Relevance of MIP-1α.

a) Stem cell inhibition.

Since *in vivo* animal studies had suggested that MIP-1α may be able to protect the haemopoietic stem cell compartment from the cytotoxicity of chemotherapy (Dunlop et al 1992; Lord et al 1992), this chemokine has been investigated in Phase I and II clinical studies. Phase I studies have shown that a non-aggregating form of MIP-1α, BB10010, is safe and is able to reduce the cycling status of marrow myeloid progenitors (Broxmeyer et al 1998). This effect was reversible. However a randomised Phase II study has suggested that this MIP-1α variant does not appear to alter the rate of bone marrow recovery following high dose chemotherapy (Bernstein et al 1997). MIP-1α also appeared to have no effect on the incidence and severity of mucositis. This study therefore appears to suggest that MIP-1α does not protect either haemopoietic or epithelial stem cells from the toxicity of high dose chemotherapy. However since full-length MIP-1α does not inhibit epithelial stem cell proliferation it is perhaps not surprising that this study has not shown any evidence of epithelial stem cell protection. Further *in vivo* studies need to be performed using the kerato-inhibitory form of MIP-1α to determine whether this molecule can protect epithelial stem cells from the toxicity of cancer chemotherapy.

b) MIP-1α and inflammatory disease.

A number of studies have shown that chemokines are potent pro-inflammatory molecules, and that the the primary receptor-binding domain of these proteins is the amino-terminus (Reviewed by Baggiolini et al 1997). Several studies have also shown that the chemokines are naturally modified by amino-terminal truncation, and that this structural modification results in major functional modulation. Amino-terminal truncation often results in the generation of potent chemokine antagonists which may be able to function as anti-inflammatory molecules. These may be therapeutically useful in the clinic in the treatment of inflammatory diseases such as arthritis (Gong et al 1997). It would therefore be interesting to study the *in vitro* inflammatory profiles of the amino-terminally truncated MIP-1α mutants generated in these studies. If these

proteins exhibit any anti-inflammatory activity, then further analyses using *in vivo* models should be performed to evaluate the therapeutic potential of these molecules.

c) MIP-1α and Carcinogenesis.

The studies presented in this thesis suggest that a structurally altered form of MIP-1α is able to inhibit keratinocyte proliferation. Other studies in this laboratory have shown that this COS 7 MIP-1α is not able to inhibit the proliferation of a number of human squamous cell carcinoma cell-lines (K. Parkinson: personal communication), although interestingly TGF-β1 was still able to inhibit some of these cell-lines. These studies appear to suggest that the transformation of normal keratinocytes involves loss of responsiveness to the inhibitory activity of MIP-1α. They also suggest a role for MIP-1α in carcinogenesis similar to the role previously suggested in the development of chronic myeloid leukaemia (Eaves et al 1993). Further studies into the signal transduction pathways of COS MIP-1α in keratinocytes and their derangement in squamous cell carcinomas may reveal as yet unrecognised alterations in the cellular machinery. These may then be manipulated with either specific signal transduction regulators or perhaps through targetted gene therapy.

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