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Abstract

In a multicellular organism the fate of cells is regulated by the surrounding environment. One important example of such a regulation is the development of blood cells. This complex process during which all type of blood cells are produced from a common pluripotent precursor cell is named hematopoiesis. In the adult mammals hematopoiesis is restricted to the extravascular compartments of the bone marrow, where primitive hematopoietic cells and their progeny develop in intimate contiguity with bone marrow microenvironment. Heterogeneous populations of stromal cells and components of extracellular matrix (ECM) have been identified as a part of this microenvironment. A crucial component of the ECM in bone marrow is hyaluronic acid (HA) which is instrumental for the organisation of ECM. Moreover HA can act as a signalling molecule regulating cellular functions.

This work is based on the observation that in an *in vitro* hematopoietic system, the so-called Long Term Bone Marrow Culture (LTBMC), HA addition enhances the production of progenitor and mature cells by inducing the production of the crucial hematopoietic cytokine IL-6 in Bone Marrow Derived Macrophages (BMDM).

To gain insight into the mechanisms of HA-regulated cytokine production, I established a system consisting of the progenitor cell line TF-1 and the hematopoietic supportive stromal cell line MS-5. HA triggers the production of cytokines IL-6, GM-CSF and IL-4 in MS-5 cells. I found HA regulates production of the IL-6 cytokine via the RAF-MEK-ERK signal transduction pathway. HA-induced production of IL-6 is regulated on the transcriptional level, via NF-κB and NF-IL-6 binding-sequences in the IL-6 promoter.

The TF-1 progenitor cell line also induces the production of cytokines in the MS-5 cells. Furthermore, the induction of IL-6 by TF-1 cells is also regulated on the transcriptional level and is mediated by NF-κB and NF-IL-6 cis-elements in the IL-6 promoter.

Taken together these data explore the mechanism of regulated cytokine production by stromal cells and underline the importance of ECM-cell and cell-cell interactions in hematopoiesis.

Hyaluronsäure induziert Zytokine in Stromazellen welche Hämatopoiese unterstützen

Zusammenfassung

In einem multizellulären Organismus wird das Zellschicksal durch Umgebungseinflüsse reguliert. Ein wichtiges Beispiel für eine solche Regulation ist die Entwicklung von Blutzellen. Dieser komplexe Prozeß, bei dem alle Arten von Blutzellen aus einer gemeinsamen pluripotenten Vorläuferzelle produziert werden, wird als Hämatopoiese bezeichnet.
In erwachsenen Säugetieren ist die Hämatopoiese auf die extravasalen Kompartimente des Knochenmarks beschränkt, wo einfache hämatopoietische Zellen und ihre Abkömmlinge sich in enger Einbindung in der Mikroumgebung des Knochenmarks entwickeln. Heterogene Populationen von Stromazellen und Komponenten der extrazellulären Matrix (ECM) sind als Bestandteile dieser Mikroumgebung identifiziert worden. Ein entscheidender Bestandteil der ECM des Knochenmarks ist Hyaluronsäure (HA), die wesentlich an der Organisation der ECM beteiligt ist. Darüber hinaus kann HA als Signalmolekül funktionieren, welches zelluläre Funktionen reguliert.
Die hier vorliegende Arbeit basiert auf der Beobachtung, daß HA in einem *in-vitro* System, der sog. Langzeit-Knochenmarkskultur (LTBMC), die Produktion von Vorläuferzellen und reifen Zellen erhöht, indem es die Produktion des entscheidenden hämatopoietischen Zytokins, IL-6, in den vom Knochenmark abgeleiteten Makrophagen (BMDM), induziert.
Um Einsicht in die Mechanismen der HA-regulierten Zytokin-Produktion zu erhalten, habe ich ein System mit der hämatopoietischen Vorläufer-Zelllinie TF-1 und der supportiven Stromazelllinie MS-5 etabliert. HA löst die Produktion der Zytokine IL-6, GM-CSF und IL-4 in MS-5 Zellen aus. Ich fand heraus, daß HA die Produktion des Zytokins IL-6 über den Raf-MEK-ERK Signaltransduktionsweg reguliert. Die HA-induzierte Produktion von IL-6 wird auf transkriptioneller Ebene über NF-κB und NF-IL-6 Bindesequenzen im IL-6-Promotor reguliert.
Die TF-1 Vorläufer-Zelllinie induziert ebenfalls die Produktion von Zytokinen in MS-5 Zellen. Weiterhin wird die Induktion von IL-6 durch TF-1 Zellen ebenfalls auf transkriptioneller Ebene durch NF-κB und NF-IL-6 cis-Elemente im IL-6 Promotor reguliert.
Zusammengenommen zeigen diese Daten die Mechanismen der durch Stromazellen regulierten Zytokin-Produktion und unterstreichen die Bedeutung von ECM-Zell- und Zell-Zell-Interaktionen während der Hämatopoiese.

Contents

1 Introduction

1.1 The extracellular matrix component hyaluronic acid (HA)

The development and function of all cells in a mammalian organism depend on interactions with molecules in their environment. The classes of molecules in this environment represent growth factors, cell adhesion molecules and components of the extracellular matrix (ECM). In a multicellular organism the ECM controls homeostasis, cell growth and differentiation. The ECM is a complex and highly diverse structure, including many different components such as proteoglycans, glycosaminoglycans, fibronectin, collagen, laminin, hemonectin, thrombospondin etc. Glycosaminoglycans (GAGs) are negatively charged hexosamine containing polysaccharides. They are ubiquitously present in the ECM of connective tissues, in body fluids and can be also found on the cell surface.

In the last years, glycosaminoglycans have been intensively studied because of their prominent role in tissue homeostasis and in pathological disorders. In this work we would like to introduce one member of the GAG family - hyaluronic acid (HA).

1.1.1 Molecular properties of HA

HA was originally isolated and described by Karl Meyer as the major glycosaminoglycan of the vitreous body of the eye and of the synovial fluid (Meyer *and* Palmer, 1934; Meyer *et al.,* 1947). Later on, HA was also found in a number of other tissues such as the umbilical cord, the skin, the rooster comb and the cartilage. HA is the largest ECM component with a molecular mass of several million daltons. It is a linear polymer composed of disaccharide repeats that consist of N-acetylglycosamine and D-glucuronic acid (Fig.1) (Laurent, 1989).

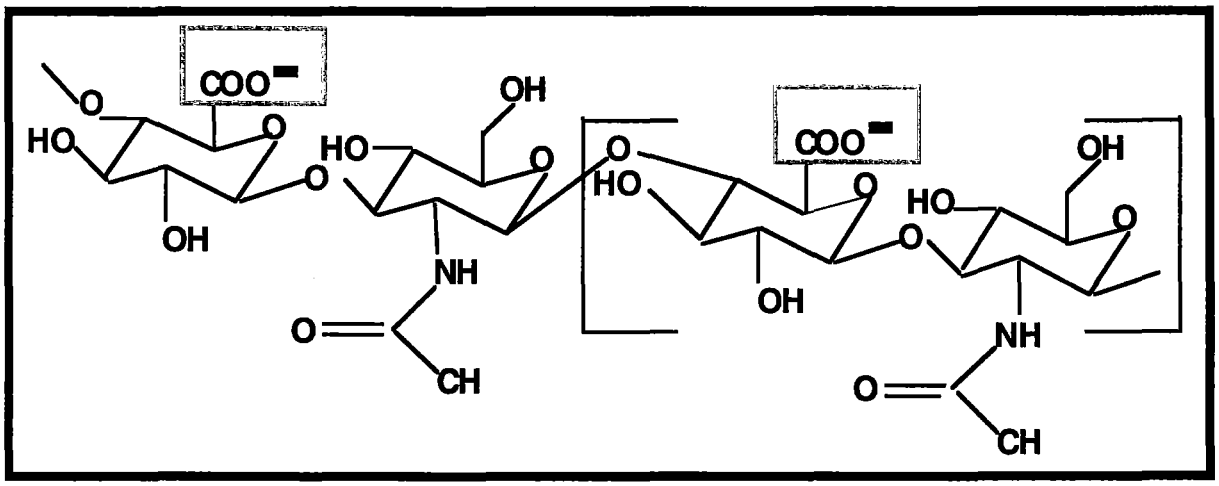


Figure 1. The structure of HA disaccharides.

Because of its charged residues HA can adopt many water molecules and thus is able to expand into tissue spaces and to occupy an area of several magnitudes larger than expected from its size. The hydratation properties of HA are important for creation of an environment that maintains homeostasis and facilitates cell movement.

In solution the HA molecules form three-dimensional structures, which show extensive intramolecular hydrogen bonding. This restricts the conformation flexibility of the polymer chains and induces distinct secondary (helical) and tertiary (coiled coil) structures (Evered *and* Whelan *(eds)*, 1989). Because of such an organisation, HA behaves in aqueous solutions as an unusually stiff polymer. This rigidity is important for the space-filling role of HA and also for the formation of specific channels through which different water-soluble molecules can diffuse.

Another important feature of HA is that it interacts with proteoglycans and other matrix components, which use the HA network in order to associate and orientate themselves. Thus, HA organises the macrostructure of the ECM and provides stability and elasticity to the organism (Knudson *and* Knudson, 1993), for example as a central structural unit in cartilage or as a lubricant in sinovial fluid. However, it was recently discovered that HA has a variety of other

important functions. It influences cell differentiation, motility, proliferation and migration and thus affects cellular fate in the embryo as well as in the adult organism (for review see Evered *and* Whelan *(eds),* 1989). In embryogenesis HA plays a crucial role as it surrounds the proliferating and migrating cells of developing tissues. The presence of HA from the very beginning of the development of the embryo (Brown *and* Papaioannou, 1993) underlines the importance of this molecule in a variety of decisive events ongoing in embryogenesis.

For example, HA induces condensation of mesenchymal cells, that results in the onset of chondrogenesis and myogenesis (Knudson, 1993). HA has also been shown to promote migratory and proliferative events during the maturation of the nervous system (Erickson *and* Perris, 1993) and to take part in the brain development (Bignami *et al.,* 1993; Marret *et al.,* 1994). HA is the major glycosaminoglycan of the early cardiac ECM during the heart development. The strongest evidences for a function of HA in the development of the heart came from the observation that degradation of HA caused abnormal formation of the heart structure (Baldwin *et al.,* 1994). The general importance of HA in embryonic development is emphasised by the observation that abrogation of HA synthesis in knock out mice leads to the early death of the embryo.

In the adult organism, in addition to the functions already mentioned above HA is specifically involved in hematopoiesis and in the function of the immune system (Minguell, 1993; Naor *et al.,* 1997; Siegelman *et al.,* 1999). HA also contributes to the motile responses and migration of cells during wound repair (Weigel *et al.,* 1986; Weigel *et al.,* 1988; Weigel *et al.,* 1989).

HA is also important in pathological processes such as inflammation and tumorigenesis. Increased levels of HA in the serum have been found in inflammatory rheumatic diseases such as rheumatoid arthritis, scleroderma and psoriatic arthritis (Engstrom-Laurent *et al.,* 1985; Dahl *et al.,* 1985). The regulatory role of HA in inflammation was further confirmed by the observation that HA stimulates phagocytosis in monocytes and granulocytes and that HA mediates lymphocyte activation (Hakansson *et al.,* 1980; Ahlgren *and* Jarstrand, 1984; Bourguignon *et al.,* 1993; Galandrini *et al.,* 1994; de Grendele

et al., 1996). Interestingly, HA like other GAGs have important functions in the regulation of the activity of the inflammatory cytokine IFN-γ *in vivo* by direct binding and presentation of IFN-γ molecules to the cells (Fernandez-Botran *et al.,* 1999).

The importance of HA for cell migration suggests a role for HA in tumour invasion (Knudson 1996; Knudson *et al.,* 1989). Indeed, some solid tumours contain high concentrations of HA that correlate with their invasiveness (Menzel *and* Farr, 1998; Hopwood *et al.,* 1974; Philipson *and* Schwartz, 1984).

Interestingly, some tumour cells were shown not only to produce HA on their own but also to stimulate other cell types to increase HA production (Knudson *et al.,* 1984; Knudson *and* Knudson, 1990; Asplund *et al.,* 1993).

1.1.2 HA synthesis

The regulation of HA synthesis and catabolism is crucial for its functions and is co-ordinately controlled in mammalian organisms. Usually, the synthesis of GAGs occurs intracellularly. It involves elongation of sugar chain residues to core proteins followed by modifications in the Golgi apparatus and their transport in vesicels to the cell surface. In contrast, the synthesis of the enormously huge HA molecule occurs in the plasma membrane (Prehm, 1989). The HA synthase (HAS) synthesises HA residues at the inner surface of the plasma membrane and the growing HA chain is then extruded through the membrane directly into the extracellular space.

The HA synthesis correlates with the stages of embryonic development, with tissue remodelling and cellular multiplication processes in the adult organism (Teder *et al.,* 1995). HA synthesis was also found to be significantly increased during inflammation and cancer (Laurent, 1989). The elevation of HA in these processes is regulated by growth factors or/and other mediators that can stimulate HA synthesis. Indeed, several factors were shown to be involved in the regulation of HASes activity such as TGF-β, TNF, IFN-γ, PDGF, IGF-1, IL-1 (Haubeck *et al.,* 1995; Heldin *et al.,* 1989; Heldin *et al.,* 1992; Honda *et al.,* 1991; Sampson *et al.,* 1992; Ito *et al.,* 1993).

1.1.3 Catabolism of HA

HA exists as a high molecular weight polymer, but it can be degraded during different physiological and pathological processes. In mammals, three groups of enzymes degrade HA into its monosaccharides components, namely hyaluronidases (HA«ase), β-D-glucuronidases and β-N-acetyl-D-hexosaminidases. Hyaluronidases are exclusively involved in the degradation of HA and connective tissue polysaccharides, whereas the exoglycosidases (β-D-glucuronidase and β-N-acetyl-D-hexosaminidase) participate in addition in the catabolism of other substrates. There are several groups of hyaluronidases which can specifically degrade HA, using different reaction mechanisms (Csoka *et al.*, 1997).

The mammalian forms are endohexosaminidases that randomly reduce the size of the polymers through a range of oligosaccharides to finally yield tetrasaccharide residues. As we will discuss in the following chapters, for a number of HA-mediated functions the size of the polymer plays a crucial role.

The turnover of HA in the body varies in different tissues according to its structure and to the stage of maturity. HA is transported in the lymphatic vessels to lymph nodes for degradation or is catabolised locally in tissues (Fraser *et al.*, 1983; Fraser *et al.*, 1988; for review see Menzel *and* Farr, 1988). The lymph nodes extract nearly 50-90% from the peripheral lymph system. HA can also be removed directly from the blood and the lymph circulation by endothelial cells of the liver sinusoids. In tissues macrophages, chondrocytes, fibroblasts and epidermal cells take part in HA degradation. The catabolism of HA is mediated via receptor-mediated up-take. Two of such receptors have been described: one is CD44, which plays this role in fibroblasts and alveolar macrophages (Culty *et al.*, 1992; Culty *et al.*, 1994; Hua *et al.*, 1993) Another one was identified on liver endothelial cells LEC (Yannarielo- Brown *et al.*, 1992). In addition non-specific endocytosis or/and binding to low affinity receptor can also take part in HA uptake.

1.1.4 HA-binding proteins

HA-binding proteins can be separated in two groups according to the structure of their HA-binding region. In both cases, the respective proteins have defined HA-binding motifs which are characterised by two basic amino acids flanking a region of seven amino acids $B(X_7)B$, among which one at least is basic (Yang *et al.,* 1994). The first group of HA-binding proteins contain only such motifs. These proteins can bind HA under reducing as well as under non-reducing conditions. The other group of HA-binding proteins form in addition a common protein domain (link module) firstly detected in the link protein, a protein of the ECM that "links" HA to other ECM components. This module is characterised by cystein residues (in a part of HA-binding domain), which form disulphide bonds and establish a defined tertiary structure in the molecule (Goetinck *et al.*, 1987). The functional importance of this module was demonstrated by the fact that HA-binding is abrogated under reducing conditions (Toole, 1990).

Some of the HA binding proteins like the cartilage link protein, versican, aggrecan, hyaladherins bind HA within the ECM. Others are specific cell-surface HA-binding receptors.

1.1.5 HA-receptors

For many of the physiological functions of HA such as cell adhesion, growth and migration its binding to receptors is required (for review see Sherman *et al.,* 1994). The HA -receptor association is significantly elevated in situations when rapid tissue turnover and remodelling occur (Csoka *et al,* 1997). HA interactions with cell receptors also contribute to pathological processes such as wound healing and tumorigenesis (Laurent, 1989). Two such receptors namely CD44 and LIVE-1 have been identified to date. The previous description of RHAMM (receptor for hyaluronate mediated motility) and ICAM-1 (intercellular cell adhesion molecule-1) as cell-surface HA-receptors (Turley *et al.,* 1991; McCourt *et al.,* 1994) turned out to be based on artefacts (Hofmann *et al.,* 1998; McCourt *and* Gustafson, 1997).

1.1.5.1 CD44 protein family

CD44 was originally discovered by means of antibodies as a protein on leukocytes. Now we know that CD44 comprises a family of transmembrane cell surface glycoproteins type I. Members of the CD44 protein family play roles in physiological and pathological processes such as limb development, hematopoiesis, lymphocyte homing, in immune responses and cancer (for review see Lesley *et al.,* 1993a; Naor *et al.,* 1997).

Proteins of the CD44 family are widely distributed on various cells, including keratinocytes, hematopoietic cells and many others. However, the pattern of CD44 expression in the body was shown to be specific for certain cell types and for differentiation stages.

The standard form of CD44 (CD44s) consists of a stretch of 248 amino acid in the extracellular part, a stretch of 23 amino acid comprising the transmembrane part and of a stretch of 70 amino acid establishing the intracellular part. The variety of CD44 isoforms is created by alternative splicing of variant exons sequences that are all excised in the CD44s isoform (Stamenkovic *et al.,* 1991; Screaton *et al.,* 1992). The difference between CD44s and the higher molecular weight variant forms (CD44v) consists of additional peptide sequences inserted into the membrane-proximal extracellular part of the CD44s molecule. Different splice variants of CD44 are believed to have distinct physiological functions. For example, CD44 variants containing the variant exon 6 (v6) were shown to confer the metastatic potential to non- metastatic pancreatic carcinoma cells upon transfection (Herrlich *et al.,* 1993). Exon v6 containing variants are expressed on B/T lymphocytes and on macrophages upon antigenic stimulation. CD44 variants containing v7 exon sequences are involved in chronic inflammatory diseases (Wittig *et al.,* 1997). A v3 containing variant is crucial for limb development. In this case the function of the CD44 variant appeared to be the presentation of growth factors to target cells (Bennett *et al.,* 1995; Sherman *et al.,* 1998).

Many if not all functions of CD44 require binding to a ligand. CD44 can bind several molecules such as fibronectin (Jalkanen *and* Jalkanen, 1992; Toyama-

Sorimachi *et al.*, 1995) collagen, laminin (Turley *et al.*, 1984) the chondroitin sulfate modified form of the invariant chain (Naujokas *et al.*, 1993), osteopontin (Weber *et al.*, 1996) and some growth factors (Tanaka *et al.*, 1993; Bennett *et al.*, 1995; Van der Voort *et al.*, 1999). The best characterised ligand of CD44 is HA (Aruffo *et al.*, 1990; Miyake *et al.*, 1990).

The evidence that CD44 is a cell surface receptor for HA came from the observation that purified CD44 can bind HA *in vitro* and specific antibodies against CD44 block HA-binding to the cell surface. In addition, introduction of CD44 into cells that do not bind HA confer the HA binding ability (for review see Lesly *et al.*, 1993a).

CD44 has two HA-binding motifs in its extracellular domain similar to those of other HA-binding proteins (see above) (Peach *et al.*, 1993). The significance of these two motifs with respect to HA-binding was confirmed by site -directed mutagenesis. One additional motif for HA-binding was found in the cytoplasmic tail of CD44. It is functional *in vitro*, although its functional role *in vivo* is still not clear. In addition to these binding motifs, a secondary loop structure forms the link domain characteristic for HA binding (see above) in the N-terminus of CD44. The minimal size of HA molecule CD44 can interact with is six sugar disaccharides, but higher affinity is observed for longer HA molecules (Underhill *et al.*, 1983).

HA-binding of CD44 can be modulated

Many cells do not bind HA, although they express high levels of CD44, suggesting that additional activating events are necessary. Indeed, some cells can be "activated" to bind HA by various stimuli including certain CD44 antibodies. For example, the CD44 specific monoclonal antibodies (mAbs) IRAWB14 as well as antigenic stimulation can induce non HA-binding T-cells to bind HA (Lesley *and* Hyman, 1992). Cytokines regulate CD44 dependent HA binding of cells of the immune system. E.g. the cytokine TNF-α increases HA-binding of monocytes, whereas IL-4 and IL-13 decrease it (Levesque *et al.*, 1999; Levesque *and* Haynes, 1997). IL-5 stimulates CD44 dependent HA binding in B-cells (Murakami *et al.*, 1990). The bacterial component LPS

(lipopolysaccharide) was shown to stimulate CD44 mediated HA-binding in peripherial blood monocytes (Levesque *and* Heines, 1997). TPA treatment can also activate CD44 mediated HA-binding (Liao *et al.*, 1993; Sionov *and* Naor, 1998).

Cross-linking or clustering of CD44 may influence its HA binding. This can be concluded from the observation that only multivalently binding anti-CD44 antibodies enhance HA-binding ability to CD44 receptors (Lesley *et al.*, 1993b; Sleeman *et al.*, 1996; Liu *et al.*, 1997). Interestingly, in some experiments the cytoplasmic portion of CD44 also influences HA binding, since the truncation of this part results in the reduction of HA binding (Thomas *et al.*, 1992; Lesley *et al.*, 1992; Liao *et al.*, 1993; He *et al.*, 1992). However in other experimental systems it had no influence.

CD44 interacts specifically with cytoskeleton elements such as ankyrin (Bourguignon *et al.*, 1992) and actin (Lacy *et al.*, 1987) via the "linker proteins" ezrin, radixin, moesin and merlin (Algrain *et al.*, 1993; Tsukita *et al.*, 1994; Hirao *et al.*, 1996). However the significance of such interactions for HA binding is still a matter of discussion. Prevention of ankyrin binding to CD44 or disruption of actin polymerisation and actinomyosin contractions abrogates CD44-mediated HA-binding in some systems (Lokeshwar *et al.*, 1994; Galluzzo *et al.*, 1995). In contrast, there are data indicating that disruption of the cytoskeleton by itself does not affect CD44 mediated HA binding (Murakami *et al.*, 1994).

The HA-binding ability of CD44 proteins is dependent on the type of isoform expressed and on the post-translation modifications. For example, the presence of splice variants on human T-cells leads to an increase in HA-binding as compared to cells expressing only the standard form. Moreover, activated human T-cells transiently express v6 and v9 CD44 variants, and their expression is important for adhesion of these cells to HA (Galluzo *et al.*, 1995). Some CD44 isoforms also play critical roles in mediating HA dependent adhesion in dendritic cells (Haegel-Kronenberger *et al.*, 1998).

CD44 isoforms differ in the repertoire of ligands they interact with. E.g. inclusion of peptide sequences encoded by the v6 and the v7 exons extends the

repertoire of GAGs, to which CD44 can bind, to chondroitin sulfate, heparin and heparin sulfate as well if CD44 is solubilised or it is expressed on the cell surface (Sleeman *et al.*, 1997).

The importance of CD44 posttranscriptional modifications for HA binding was demonstrated by experimental modifying of CD44. CD44 has potential glycosylation and phosphorylation sites and can also undergo glycanation. Sulfatation and addition of chondroitin sulfate are required for HA-binding by CD44 in some cells (Esford *et al.*, 1998). Keratin sulfate modification of CD44 has an inhibitory effect on its HA binding (Takahashi *et al.*, 1996).

The glycosylation status of CD44 is important for its HA-binding ability (Katoh *et al.*, 1995; Bartolazzi *et al.*, 1994; Skelton *et al.*, 1998). Different pattern of glycosylation can regulate HA-binding in both a positive and a negative manner. Whereas the inhibition of N-linked glycosylation of CD44 does not influence HA-binding in colon carcinoma cells, the inhibition of O-glycosylation can significantly enhance it (Dasgupta *et al.*, 1996). In activated B-lymphocytes, abrogation of N-glycosylation leads to increased HA-binding (Hathcock *et al.*, 1993).

However, in hematopoietic progenitor cells and in CD44v4-v7 transfected pancreatic tumour cells the contrary result was observed. When N-glycosylation was reduced HA-binding was decreased (Sleeman *et al.*, 1996; Moll *et al.*, 1998). The importance of phosphorylation of the CD44 cytoplasmic tail for HA-binding in a murine system was shown by using CD44 mutants defective in phosphorylation. The abrogation of phosphorylation of the serine residues 325, 327 led to the reduction of HA-binding (Pure *et al.*, 1995). However, human cells transfected with such defective CD44 mutants were still able to bind HA (Uff *et al.*, 1995).

Taken together, different factors such as changes in glycosylation, distribution of CD44 on cell surface and modification of the cytoplasmic domain of CD44 contribute to the regulation of CD44 mediated HA-binding.

1.1.5.2 LIVE-1

LIVE-1 (Lymphatic vessel endothelial HA receptor 1) seems to be the major HA receptor on lymph vessel endothelia (Banerji *et al.*, 1999). It was shown to bind both immobilised and soluble HA. This receptor, similarly to CD44, is a member of the link protein superfamily. LIVE-1 consists of 322 aminoacids and shows the features of an integral membrane protein type I. LIVE-1 has a hydrophobic domain of 212 amino acids with seven cystein residues, a serine/threonin-rich region and two motifs for N-linked glycosylation in the extracellular domain. LIVE-1 has 41% of homology with CD44. The region of the highest homology with CD44 is located in the area of the link domain.
It is speculated that LIVE-1 functions on the surface of endothelial cells as an endocytic receptor like CD44 on macrophages and might be as well involved in the migration of immune cells.

1.1.6 HA as a signalling molecule

Through its interactions with cellular receptors HA can act as a signalling molecule in various systems. For example, HA induces tyrosine phosphorylation of several proteins in bovine aortic endothelial cells (Slevin *et al.*, 1998), increases tyrosine phosphorylation of two proteins (m.w. 124 kDa and 60 kDa) in NIH 3T3 cells (Moon *et al.*, 1998) and triggers Ca^{2+} -dependent signal transduction cascade in T-lymphocytes followed by cytoskeleton rearrangement (Bourguignon *et al.*, 1993). HA mediated-activation of Mitogen-Activated Protein Kinases (MAPKs) was also observed in several cell types (Slevin *et al.*, 1998; Khaldoyanidi *et al.*, 1999).
HA-induced signal cascades result in regulation of expression of genes and thereby regulate cellular fate and/or tissue morphogenesis. One consequence of HA signalling is the induction of proliferation observed in the aortic endothelial cells and in NIH3T3 cells similar to the action of growth factors (Moon *et al.*, 1998). Interestingly, in primary fibroblasts HA regulates proliferation by increasing tubulin expression, which, in turn, drives cells through the cell cycle and thus promotes cell division (Greco *et al.*, 1998).

An important example of the role of HA in morphogenesis is its regulation of neo-vascularisation. Interestingly, the size of HA is crucial for this process. For example, neo-vascularisation during wound healing is inhibited by high-molecular weight HA, but is induced by shorter HA products (Lees *et al.*, 1995). Also low molecular weight products of HA degradation, but not native high molecular weight HA, are able to induce angiogenesis *in vivo* (Deed *et al.*, 1997). In agreement with this observation, HA derived oligosaccharides (o-HA) were reported to modulate the invasive properties of bovine microvascular endothelial cells and synergise specifically with VEGF in the induction of angiogenesis (Montesano *et al.*, 1996). Another role of low molecular weight HA in tissue morphogenesis is the induction of vascular tube formation by brain endothelial cells (Rahmanian *et al.*, 1997).

Most importantly, HA can trigger signals stimulating and regulating the expression of a number of genes crucial for the immune response and function of hematopoietic system. In both systems HA can stimulate the expression of chemokines and cytokines in a variety of cell types such as macrophages, monocytes, T-cells, dendritic cells (Hiro *et al.*, 1986; Noble *et al.*, 1993; Galandrini *et al.*, 1994; Haegel-Kronenberger *et al.*, 1998; McKee *et al.*, 1996). E.g. rabbit peritoneal macrophages and human peripheral monocytes as well as murine macrophages and dendritic cells produce IL-1β upon HA stimulation (Hiro *et al.*, 1986). The induction of IL-1β cytokine might also explain the role of HA in pathological and physiological processes in connective tissues. In the inflammatory response HA induces in addition other types of inflammatory molecules. E.g. in dendritic cells HA triggers the expression of IL-8, IL-10 and IL-12 cytokines that then regulate the maturation and function of immune cells (Haegel-Kronenberger *et al.*, 1998).

HA fragments can induce the metalloelastase (MME) production in mouse alveolar macrophages. MME is implicated in different lung disorders. Therefore HA fragments, which usually accumulate at sites of lung inflammation, may trigger the expression of MME by macrophages resulting in lung inflammation (Horton *et al.*, 1999). Several other genes of the inflammatory response such as MIP-1α, the cytokine responsive gene-2 and the monocyte chemoattractant

protein-1 were identified to be induced upon treatment of macrophages with HA-fragments (McKee *et al.,* 1996). In human alveolar macrophages from patients suffering from idiopathic pulmonary fibrosis IL-8 mRNA was also significantly increased upon HA-fragments treatment (McKee *et al.,* 1996). In alveolar macrophages HA also induces the expression of inducible nitric oxide synthase (INOS), another molecule involved in the inflammatory response. HA fragments can induce INOS expression on their own, but the expression of the INOS gene is significantly increased when HA synergizes with INF-γ (McKee *et al.,* 1997).

In hematopoiesis HA has an enhancing effect on the production of blood cells *in vitro,* that is due to the increased production of IL-6, one of the crucial hematopoietic cytokine, by bone marrow derived macrophages (BMDM) (Khaldoyanidi *et al.,* 1999). Dendritic cells respond to HA also by upregulation of cytokines relevant for hematopoiesis such as IL-6 and GM-CSF (Haegel-Kronenberger *et al.,* 1998).

1.2 Hematopoiesis

Throughout their lifespan all mammals continuously produce different types of blood cells such as monocytes/macrophages, red cells, platelets, T and B lymphocytes and neutrophils in a process called hematopoiesis.

These cells all originate from common pluripotent hematopoietic stem cells (HSCs). The HSCs are the only cells that are able to fully reconstitute the hematopoietic system of animals in which all hematopoietic cells have been killed upon irradiation. In addition to their differentiation capacity, HSCs are capable of self -renewal.

The HSCs first differentiate into progenitor cells, which are committed to the various hematopoietic lineages. Therefore, in a hierarchical order, the HSCs initiate the production of more and more specialised cells (Fig.2) (Metcalf, 1989). The final mature cells entering the blood are fully differentiated. The majority of mature blood cells are short-lived and incapable of further division.

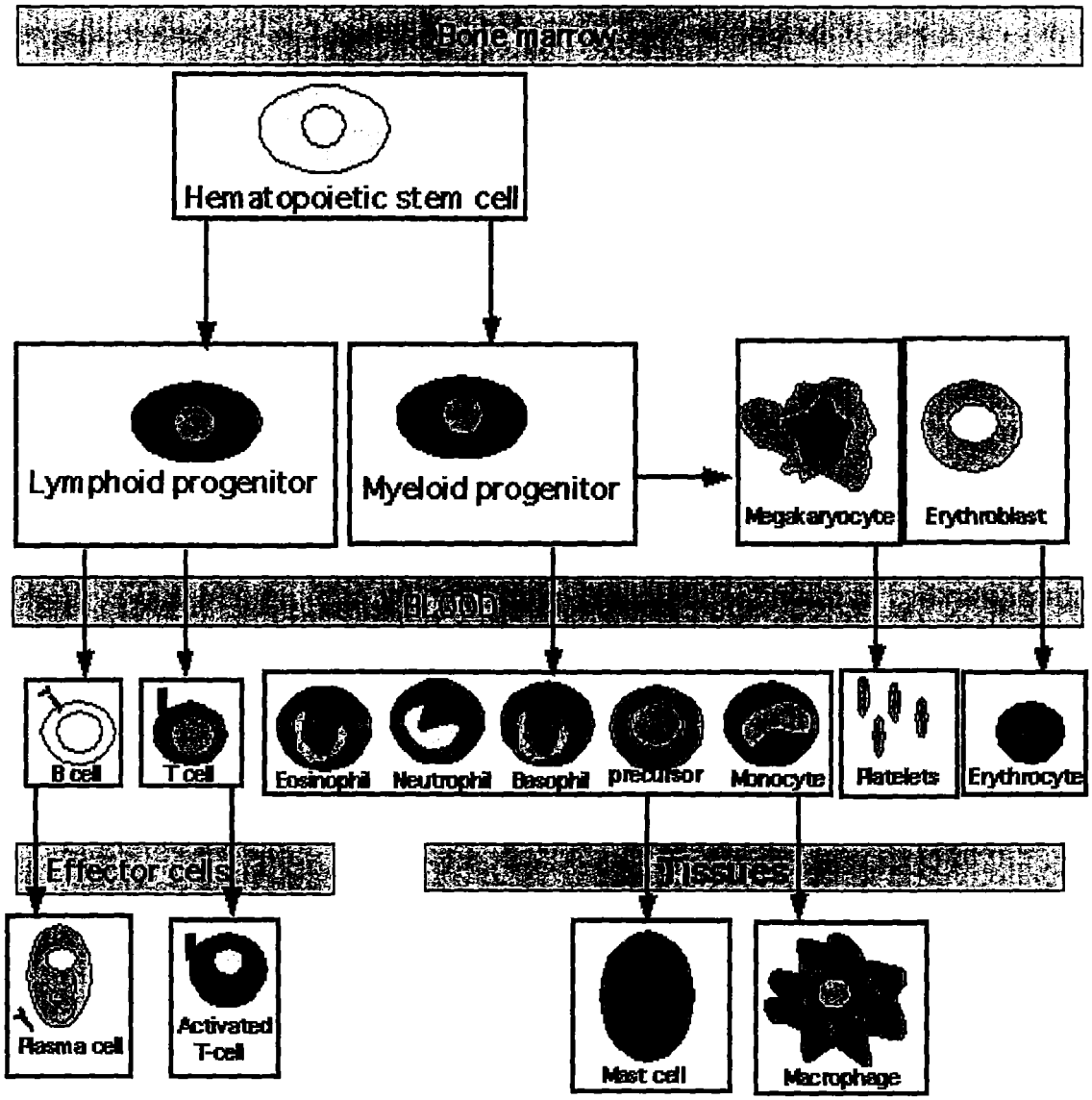


Figure 2. The hierarchy of the hematopoietic system.

1.2.1 Embryonic development of hematopietic system

The pluripotent HSCs do not develop in the embryo from the very beginning. Firstly, a primitive hematopoitic system is established in the embryo composed of primitive hematopoetic precursors for the erythro-myeloid, granulocyte-macrophage and lymphoid lineages. The definitive (adult -type) HSCs, capable of full multilineage repopulation are detected in the embryo only at later stages (Dzierzak *et al.*, 1998).

The hematopoietic system develops during embryogenesis in several tissues such as the yolk sac, the aorta-gonad-mesonephros (AGM), the paraaortic splanchnopleura (PAS), the liver, the spleen and the thymus. The earliest hematopoietic activities are observed in the yolk sac and in the intraembryonic PAS/AGM region. These tissues develop from the mesodermal germ layer cells formed in the gastrulating embryo. The molecular mechanism of hematopoietic specification within the mesoderm involves the action of various factors of the TGF-β superfamily and the FGF family (Smith *and* Albano, 1993; Dale *et al.*, 1992; Mead *et al.*, 1996; Turpen *et al.*, 1997). In particular BMP-4 (bone morphogenesis protein), a member of the TGF-β family, and its receptor BMP-2/4 play a crucial role in this early transition step. This was convincingly shown in BMP-4 knock out embryos the majority of which die around gastrulation with little or no mesoderm differentiation and the surviving embryos have profound defects in the yolk sac mesoderm (Winnier *et al.*, 1995). Further support for a decisive role of BMP-4 in hematopoiesisis originates from the observation that BMP-4 can induce hematopoietic differentiation of pluripotent embryonic stem cells (Johansson *and* Willes, 1995).

Transcription factors have also been identified that are crucial for the development of the embryonic hematopoietic system. The transcription factor Tal-1/SCL (T-cell leukaemia oncoprotein/ stem cell leukaemia) is thought to be the "master" gene inducing embryonic hematopoiesis and formation of HSCs. Tal-1/SCL-/- mice do not establish embryonic erythropoiesis within the yolk sac and develop no myeloid progenitors (Shivdasani *et al.*, 1995a). In addition, Tal-1/SCL-/- mice are also unable to generate definitive HSCs (Porcher *et al.*, 1996).

The PAS/AGM region is the primary tissue developing definitive HSCs, which colonise later the yolk sac. However, this does not exclude further colonisation of the PAS/AGM region by the yolk sac cells (Fig.3). The yolk sac and the intraembryonic PAS/AGM region represent two "pre-liver" stage tissues for hematopoiesis. Later, at embryonic day 9 to 11, the hematopoietic progenitors from these tissues inoculate the liver, which is the major hematopoietic tissue during the fetal stage. Fetal liver HSCs undergo daily self-renewing divisions

and are mobilised around embryonic day 15 to colonise the bone marrow and the spleen (Fig.3) (Morrison *et al.*, 1995).

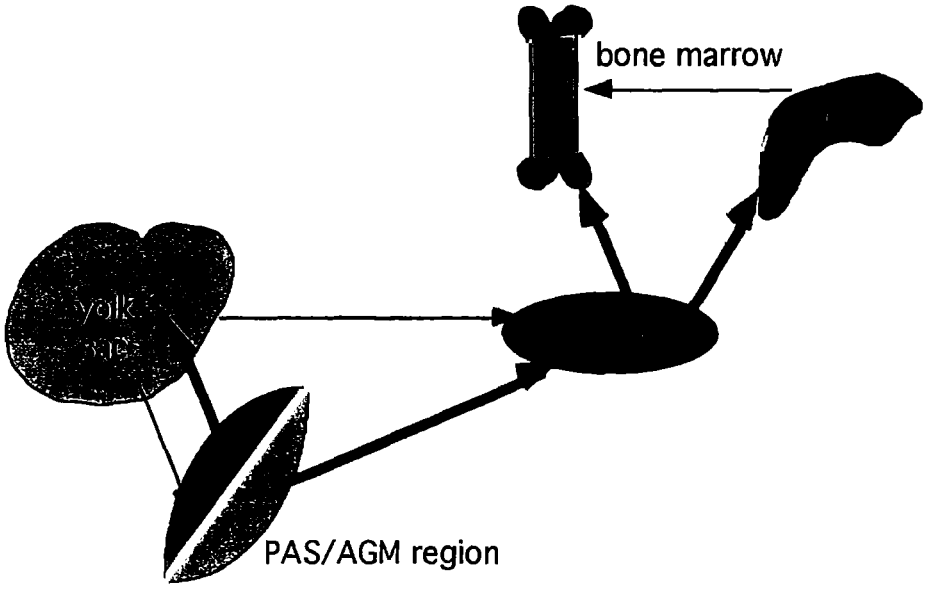


Figure 3. Sequence of hematopoietic colonisation events in the developing mouse embryo. *Bold black arrows represent general colonisation events; black arrows represent additional colonisation events.*

Shortly before birth, in the last stages of embryogenesis, and during the adult life the bone marrow becomes the major site of hematopoiesis.

1.2.2 Hematopoiesis in adult mammals.

The processes of self-renewal and differentiation of pluripotent stem cells are maintained almost throughout the adult life span, but seem to cease at high age. The reduction of the proliferative capacity could be caused by a shorting of the telomeres in pluripotent stem cell upon more and more divisions (Vaziri *et al.*, 1994). Compatible with a function of telomers in determining the proliferation potential of stem cells is also the finding that self-renewing mouse hematopoietic cells, unlike non-self-renewing cells, express telomerase, an enzyme that reconstitutes telomere ends (Morrison *et al.*, 1996).

The major steps of differentiation of HSCs take place in special sites of the bone marrow. Stem cells can circulate in the blood and can re-enter the marrow and re-establish hematopoiesis in the marrow cords. Thereby retention and migration of stem cells and progenitors within bone marrow are important features of hematopoiesis.

Primitive hematopoietic precursors express a variety of cell adhesion molecules (CAMs) implicated in the regulation of retention and migration such as the integrin VLA-4 (for very late antigen), the member of sialomycin family CD34, the immunoglobulins CD31, CD50 and the ligands for selectin (Simmons *et al.*, 1997). In particular, the interaction between the integrin VLA-4 expressed on HSCs and VCAM-1 expressed in the bone marrow stroma is an important key in the HSC retention and migration (Miyake *et al.*, 1991). The importance of the VLA-4 integrin molecule for retention is confirmed by the fact that antibodies directed against this integrin can efficiently mobilise hematopoietic progenitors (Yang *et al.*, 1995; Hirsch *et al.*, 1996).

Immature hematopoietic progenitors receive from the bone marrow microenvironment a number of stimuli for further differentiation which include membrane- bound, soluble or ECM- associated cytokines, ECM components and adhesion molecules. Under the influence of these stimuli the early hematopoietic progenitors switch their transcriptional programs, which then determine the subsequent fate of the cells (Hu *et al.*, 1997). Several lineage-restricted transcription factors have already been identified as necessary for the hematopoietic development of particular lineages (Shivdasani *and* Orkin, 1996). For example GATA-2 and c-Myb are implicated in the maintenance and the expansion of most early progenitors (Tsai *et al.*, 1994; Mucenski *et al.*, 1991). The transcription factor giving rise to erythroid differentiation is GATA-1 (Tsai *et al.*, 1989; Evans *and* Felsenfeld, 1989).

The transcription factors C/EBPβ (also called NF-IL-6) and PU.1 were shown to regulate a large number of myeloid specific genes (Scott *et al.*, 1994; Zhang *et al.*, 1994). Ikaros, Pu-1, Pax-5 and E2A seem to be responsible for T and B lymphoid lineage differentiation (Cross *and* Enver, *et al.*, 1997; Georgopoulos *et al.*, 1994; Urbanek *et al.*, 1994; Zhuang *et al.*, 1994). NF-E2 was reported to

be specific for the megakaryocytic development (Shivdasani *et al.*, 1995b). The transcription factor NF-κB plays an important role in B cells maturation (Franzoso *et al.*, 1997).

1.2.3 The structure of bone marrow

Although in the adult organism hematopoietic precursors can be found in a number of tissues, the bone marrow is the only tissue in which myelopoiesis, lymphopoiesis and erythropoiesis occur simultaneously (Rosse, 1976). These processes take place in the extravascular space between the bone marrow sinuses. As pictured in Fig. 4, those sinuses are organised by a sinusoidal network from the branches of artherias re-entering the marrow cavity. These sinuses collect into a large central sinus from which the blood enters the systemic venous circulation through emissary veins (De Bruyn, 1970).

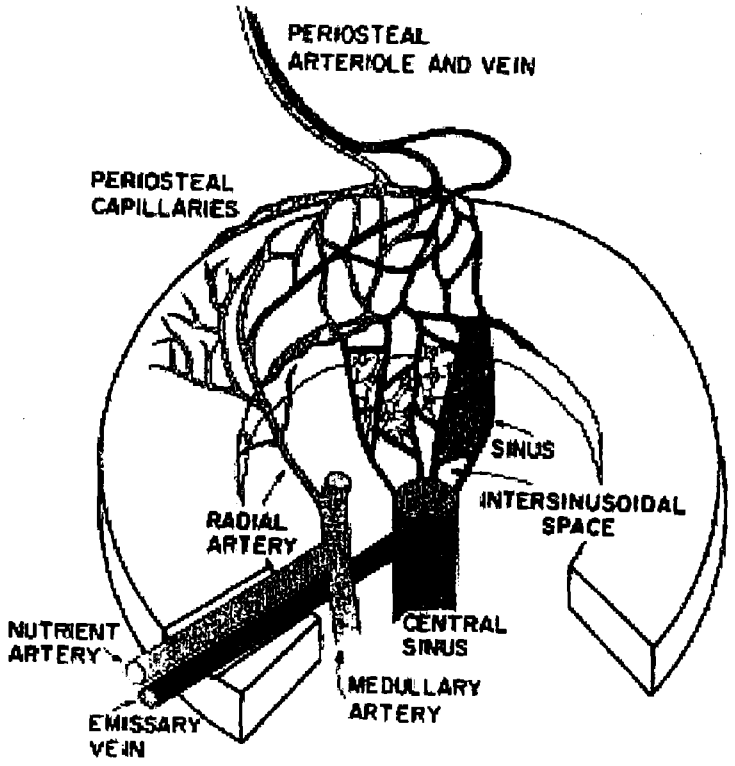


Figure 4. Cross-section of a long bone showing the medullary cavity and the marrow circulation. *Redrawn from Dorshkind.*

The development of hematopoietic progenitors strongly depends upon their interaction with the marrow environment (Verfaillie *et al.,* 1994) comprising cellular and non-cellular components. The cellular environment in the bone marrow hematopoietic spaces consists of odent stromal cells such as endothelial cells, reticular cells, osteoblasts and adipocytes with addition of macrophages and lymphocytes. These stromal cells produce components of the ECM including proteoglycans, glycosaminoglycans, laminin, collagen, tenescin, fibronectin (Gallagher *et al.,* 1983; Wight *et al.,* 1986). The ECM together with growth factors and other molecules form the non-cellular environment for progenitor cells within the bone marrow.

1.2.4 Role of the stromal cells in hematopoiesis

The contribution of stromal cells to the regulation of hematopoiesis includes cell-cell interactions, cell-matrix interactions and the production of different soluble factors.

In situ studies of the bone marrow reveal that hematopoietic cells can be found in close association with stromal cells. Moreover, the level of this association depends on the maturation stage of hematopoietic progenitors (Mauch *et al.,* 1980; Dorshkind *and* Phillips, 1982; Coulombel *et al.,* 1983). In an *in vitro* hematopoietic system called long term bone marrow culture (LTBMC) the stem cells and the earliest precursors bind tightly to the stromal cell layer, whereas more mature cells can be found in the non-adherent cell fraction of LTBMC (Dexter *et al.,* 1977; Witte *et al.,* 1987; Harrison *et al.,* 1987). In accordance with this notion, the population of $CD34^+$ cells containing predominantly immature precursor cells have greater affinity for stromal cells than the unselected pool of hematopoietic cells (Liesveld *et al.,* 1989; Verfaillie *et al.,* 1990; Andrews *et al.,* 1990; Gunji *et al.,* 1992). Furthermore, human blast-colony forming cells can be separated from more mature colony forming progenitors by using their enhanced ability to adhere to stromal cells (Gordon *et al.,* 1985 Gordon *et al.,* 1987).

Formation of blood cells is highly dependent on those complex stromal cells -

hematopoietic cells interactions. For example, the number of cells able to form colonies of differentiated cells in methylcellulose is significantly decreased in LTBMCs in which progenitors were separated from stromal cells by a membrane as compared to control cultures (Bentley, 1981). The same is also true for some stromal cell lines, which can be generated from the adherent layer of LTBMC. E.g. more colony forming hematopoietic precursors were obtained after co-culture of hematopoietic cells on preadipocyte stromal cell lines as compared to control (Kodama *et al.*, 1982; Kodama *et al.*, 1986).

The cross-talk between hematopoietic cells and stromal cells is mediated by specific receptor-ligand interactions. The previously mentioned interaction between the integrin VLA-4 on hematopoietic cells with its ligand VCAM-1 on the stromal cells is crucial for the development of cells of the murine B-lineage and human $CD34^+$ early precursors (Simmons *et al.*, 1992). α-VLA-4 antibodies which abrogate binding of $CD34^+$ cells to the stromal layer were reported to abolish lymphopoiesis and delay myelopoiesis in LTBMC (Miyake *et al.*, 1991).

The cell adhesion molecule CD44 is expressed on both hematopoietic progenitors and stromal cells and was shown to be involved in hematopoiesis (Miyake *et al.*, 1990; Rossbach *et al.*, 1996; Moll *et al.*, 1998), although the ligand for CD44 in a number of interactions between hematopoietic and stromal cells is not yet identified. Anti-CD44 mAbs inhibit hematopoiesis in both lymphoid and myeloid cultures most likely by abrogation of the attachment of progenitors to stromal cells. In support of this assumption, the same anti-CD44 mAbs or the CD44 ligand HA could prevent interaction between B-cell hybridoma and stromal cells (Miyake *et al.*, 1990). Rosette formation of erythroid progenitors, an indication of their interaction with stromal cells, seems also to be dependent on CD44. However, this effect is not mediated by HA binding (Sugimoto *et al.*, 1994).

The adhesion of hematopoietic cells to stromal cells can also be mediated and modulated by cytokines. E.g. mast cells can adhere to stromal cells via the c-kit receptor through binding to the cytokine "stem cell factor" (SCF) (Fujita *et al.*, 1989; Boswell *et al.*, 1990; Tan *et al.*, 1990), which is located on stromal cells

(Anderson *et al.,* 1990; Toksoz *et al.,* 1992). Consequently mAbs abrogating the interaction between SCF and its receptor c-kit strongly suppress myelopoiesis in LTBMC (Ogawa *et al.,* 1991; Kodama *et al.,* 1992). CD44 mediated adhesion of human hematopoietic $CD34^+$ progenitors is enhanced by cytokines such as GM-CSF, IL-3 and SCF (Legras *et al.,* 1997; Levesque *et al.,* 1995). Moreover, the cytokines IL-3, IL-6, IL-11 and SCF were shown to directly modulate the expression of cell adhesion molecules on hematopoietic progenitors (Becker *et al.,* 1999) .

1.2.5 The role of the ECM in hematopoiesis

The ECM, which is produced by stromal cells, also contributes to the differentiation and the proliferation of progenitor cells (Dorshkind., 1990; Klein, 1995; Whetton *and* Spooncer, 1998). The different ECM molecules can interact with each other and thereby provide a network which embeds the stromal cells and the hematopoietic precursors (Long, 1992). The hematopoietic cells can attach to the ECM and some ECM components are preferentially bound by cells of particular lineages (Del Rosso *et al.,* 1981; Giancotti *et al.,* 1986; Campbell *et al.,* 1987; Campbell *et al.,* 1988; Weinstein *et al.,* 1989; Campbell *et al.,* 1990).

Moreover, the components of the ECM can also bind to cytokines, growth factors and other molecules critical for hematopoiesis and compartmentalise them thereby creating niches for differentiation of hemopoietic progenitor cells. For example, O-sulfated heparin sulphate proteoglycans can bind cytokines IL-3 and MIP-1α and proteins important for hematopoiesis such a thrombospondin (Gupta *et al.,* 1998). GM-CSF can be bound by bone marrow GAGs including HA and chondroitin sulfate and presented to hematopoietic progenitors cells (Gordon *et al.,* 1987). HA as well as some other GAGs can compartmentalise IFN-γ (Fernandez-Botran *et al.,* 1999). GAGs including heparan sulfate, chondroitin sulfate, dermatan sulfate and HA can regulate the activity of several cytokines and growth factors including IL-6, C-Mpl ligand, TGF-β and platelet factor 4 (Han *et al.,* 1996).

Thus, a main contribution of the ECM in hematopoiesis might be the binding of cytokines which are thereby regulated in their activity, which in turn is important for their presentation to target cells.

1.2.6 HA in hematopoiesis.

A crucial component of the ECM in mammalian bone marrow is HA. It is instrumental for the organisation of the ECM and regulates in addition the metabolism of cell surface glycoproteins, extracellular proteins and its own synthesis.

HA is required for the adhesion of progenitor cells directly to the stromal ECM and to the stromal cells (Morimoto *et al.,* 1994; Smadja-Joffe *et al.,* 1996; Wilson, 1997; Moll *et al.,* 1998). HA can in addition upregulate the expression of several adhesion molecules on the surface of hematopoietic cells (Oertly *et al.,* 1998).

HA is involved in the regulation of differentiation and proliferation of certain lineages in the hematopoietic system. E.g. HA can inhibit the differentiation of lymphocytes into lymphoblastoid cells and this effect is dependent on the concentration and the size of HA. HA can enhance megakaryocytopoiesis by regulating the activity of several growth factors (Han *et al.,* 1996). It stimulates increased proliferation of $CD34^+$ progenitors and their differentiation into mature eosinophils via its binding to the CD44 receptor (Hamann *et al.,* 1995).

As we described in chapter 1 HA can stimulate the production of several cytokines crucial for the hematopoietic system.

1.2.7 Cytokines

Cytokines are produced by cells to influence the growth and the differentiation of the same or of neighbouring cells by interacting with their specific receptors. Their importance for hematopoiesisis is underlined by the fact that disregulation of cytokine production leads to a variety of hematological disorders such as myeloproliferative syndroms, myelodisplasias and leukemias characterised by an

uncontrolled proliferation or/and an abrogation of differentiation of immature hematopoietic cells (for review see Beutler, Lichtman, Coller, Kipps (eds), 1995)

In most instances the development of certain hematopoietic lineages is controlled by a distinct combination of those factors. E.g. growth and differentiation of the myeloid lineage are stimulated mainly by the cytokines: GM-CSF, G-CSF, M-CSF and IL-6 and the differentiation of the lymphoid lineage is predominantly controlled by IL-7, IL-4 (Dorshkind, 1990).

Some factors can also provide a negative effect on the proliferation of progenitor cells, thereby preventing proliferative disoders. One example is TGF-β which is produced by the bone marrow cells and shows an inhibitory effect on both the lymphoid and the myeloid lineages. (Ohta *et al.,* 1987; Lenfant *et al.,* 1989; Dubois *et al.,* 1990). IL-4, in addition to its stimulatory function (see above), can also inhibit development of both lymphoid and myeloid lineages directly or can induce authentic hematopoietic inhibitors (Peschel *et al.,* 1987; Jansen *et al.,* 1989; Dorshkind, 1990).

The stromal cells of LTBMC produce several cytokines. Different subpopulations of bone marrow stromal cells produce distinct sets of cytokines (for review see Deryugina *and* Muller-Sieburg, 1993). This fact explains why certain stromal cells can support differentiation and proliferation of different types of progenitor cells.

The cytokine production can be regulated by several exogenous stimuli, including cytokines themselves, growth factors, bacterial LPS and ECM components such as HA (Tanigushi, 1988). Of particular interest is the fact that HA can induce the production of one of the most crucial cytokines in the hemapoietic system namely IL-6 (Haegel-Kronenberger *et al.,* 1998; Khaldoyanidi *et al.,* 1999).

1.2.7.1 Interleukine-6 (IL-6)

The murine IL-6 gene is located on chromosome 5 (Mock *et al.,* 1989), the human IL-6 gene has been mapped to chromosome 7 (Sehgal *et al.,* 1986). Both the human and the mouse genes consist of five exons. Human IL-6 shares 65%

homology with the mouse one. Thus, human IL-6 can substitute its mouse homolog (Yasukawa *et al.,* 1987; Tanabe *et al.,* 1988; Zilberstein *et al.,* 1986). The murine protein consists of 211 amino acids (Simpson *et al.,* 1988). Depending on the different posttranscriptional modifications present in the IL-6 molecule, both mouse and human IL-6 show heterogenicity with respect to their molecular weight (between 21 and 29 kD) (May *et al.,* 1988a; May *et al.,* 1988b). IL-6 is produced by a variety of cells including endothelial cells, fibroblasts, keratinocytes, T-cells, mast cells, monocytes and macrophages. It is involved in physiological processes such as hematopoiesis as well as in pathological processes e.g. acute phase response, tumorigenesis and host defence reactions.

In hematopoiesis, IL-6 plays a crucial role at different stages. For example IL-6 induces the entry of dormant primitive stem cell into the cell cycle (Ikebuchi *et al.,* 1987; Koike *et al.,* 1988) supports the proliferation of primitive hematopoietic precursors (Suda *et al.,* 1988; Miura *et al.,* 1993), supports the proliferation of granulocyte/macrophage progenitors (Wong *et al.,* 1988), stimulates the differentiation of megakaryocytes (Ishibashi *et al.,* 1989) and is required for B cell maturation (Kishimoto *and* Hirano, 1988) and activation of T-cells (Van Snick, 1990).

IL-6 synergises with several other cytokines such as IL-1, M-CSF, GM-CSF and IL-3, e.g. with IL-1 in B-cell differentiation (Vink *et al.,* 1988) with M-CSF in macrophage formation (Bot *et al.,* 1989), with GM-CSF in the differentiation of granulocyte and monocyte precursors (Hoang *et al.,* 1988) and with IL-3 in the proliferation of multipotential progenitors (Ikebuchi *et al.,* 1987). These observations are underlined by the fact that in IL-6 deficient mice the proliferation and differention of hematopoietic progenitors are strongly decreased (Bernad *et al.,* 1994).

In acute phase response, IL-6 induces acute phase proteins (Geiger *et al.,* 1988), enhances fever (Helle *et al.,* 1988) and triggers the release of the adrenocorticotropic hormone (Marinkovic *et al.,* 1989).

Several observations suggest a role of IL-6 in tumorigenicity. For example, IL-6 enhances the growth of B-cell hybridomas, mouse plasmacytoma and

myelomas, which suggests that IL-6 may act as a tumor growth factor (Astaldi *et al.,* 1980; Nordan *and* Potter, 1986; Klein, 1990a; Klein, 1990b; Hirano *et al.,* 1992). In support for this assumption, plasmacytomas transfected with IL-6 cDNA were found to be dramatically more tumorogenic then the parental cells (Van Snick, 1990). Besides its effects on proliferation, IL-6 induces the production of matrix-metalloproteinases (MMP-2 and MMP-9) which are important for the pathogenesis of some lymphomas (Kossakowska *et al.,* 1999).
IL-6 disregulation is linked to various diseases. For example, overproduction of IL-6 is observed in proliferative diseases such as the Lennert«s T-cell lymphoma, a disease characterised by a massive macrophage infiltration of the lymphomatous tissue (Shimizu *et al.,* 1988). Disregulation of IL-6 can cause autoimmune diseases such as glomerulonephritis and plasma cell neoplasias (Hirano, 1992; Horii *et al.,* 1993). IL-6 production by malignant tumours accounts for the development of the autoimmune syndrom in cancer patients (Yoshizaki *et al.,* 1992).
IL-6 production is stimulated by viral or bacterial infections, by HA and by various cytokines such as IL-1, TNF-α, IL-3, GM-CSF, platelet derived factor (Shalaby *et al.,* 1989; Van Damme *et al.,* 1987; Kohase *et al.,* 1987; Bernasconi *et al.,* 1995; Tuyt *et al.,* 1996).

1.2.8 Experimental hematopoietic systems

In order to study mechanisms which regulate blood cell production, several systems were developed that allow to study the different steps of hematopoiesis *in vitro.*

1.2.8.1 *In vitro* differentiation of embryonic stem cells (EC)

One system corresponds to the embryonic stem cells (ES cells) grown *in vitro.* ES cells are totipotent cells derived from the inner cell mass of the developing blastocysts (Evans *and* Kaufman, 1981). When those cells are cultured on embryonic fibroblasts or in the presence of the leukaemia inhibitory factor (LIF), they retain their totipotent capacity (Williams *et al.,* 1988).

Differentiation of ES cells can be induced by several means *in vitro*. For the investigation of hematopoiesis, ES cells are cultured on stromal cells. In this case stromal cells provide the necessary microenvironment for the development of hematopoietic cells in the embryonic body (EB), which develops from the ES cells (Keller *et al.*, 1995).
The molecular mechanisms involved in the establishment of the hematopoietic system *in vivo* are also functioning in the development of the EBs (Burkert *et al.*, 1991; Schmitt *et al.*, 1991). Therefore, the ES/EB system is especially useful for defining the earliest steps of hematopoiesis, corresponding to the commitment of the precursor populations and for analysing the genes involved in this process.

1.2.8.2 LTBMC

Another system to investigate hematopoiesis *in vitro* is the so-called long term bone marrow cultures (LTBMC). According to the culture conditions, one can study myeloid or lymphoid differentiation in murine LTBMC or myeloid differentiation in LTBMC derived from human bone marrow (Dexter *et al.*, 1977; Whitlock *and* Witte 1982; Whitlock *et al.*, 1984). The composition of the medium, the temperature and the establishment of the stromal layer determine whether myelopoiesis or lymphopoiesis is triggered in the murine LTBMC.

1.2.8.3 Hematopoiesis -supporting stromal cell lines

Stromal cell lines can mimic in part the hematopoietic support provided by the stromal layer in LTBMC. Since they originate from different stromal cells such as fibroblast, preadipocytes and endothelial cells they reflect the heterogeneity of the bone marrow stroma (Deryugina *and* Muller-Sieburg, 1993). They differ in their morphology, in the production of cytokines and of ECM components. This explains their ability to support different types of hematopoietic cells. Therefore different cell lines are used to study steps of certain lineage differentiation, to examine signalling events which are leading to the activation of particular transcription factors and/or to the production of particular cytokines.

1.2.8.4 MS-5 cell line

One of such supportive hematopoitic stromal cell lines is the murine bone marrow derived stromal cell line MS-5. This cell line is derives from adherent layer of LTBMC (Itoh *et al.,* 1989) and can support the growth and proliferation of hematopoietic stem cells and progenitor cells but also of some progenitor cell lines (Suzuki *et al.,* 1992; Issaad *et al.,* 1993; Auffray *et al.,* 1994; Nishi *et al.,* 1997; Berthier *et al.,* 1997). The supportive property of MS-5 cells can be explained by the production of a number of molecules critical for hematopoiesis. MS-5 cells produce the ECM components fibronectin, laminin, collagen type 1, heparin sulfate and chondroitin sulfate proteoglycans (Drzeniek *et al.,* 1997) and basal levels of cytokines such as IL-6, GM-CSF and SCF (Suzuki et al., 1992). MS-5 cells also produce stromal cell- derived factor 1 (Bleul *et al.,* 1996), which was previously implicated in the direct migration of lymphocytes and nerve growth factor, which together with SCF is required for the proliferation of the human progenitor cell line UT-7 on MS-5 cells (Auffray *et al.,* 1996).

To trigger hematopoiesis, a close contact between the MS-5 cells and progenitor cells seems to be essential. In some instances such a close contact is mediated via HA binding, e.g. for the myeloid KG562 cell line (Moll et al., 1998). For another cell line ELM-I-1 cell-cell contact with MS-5 cells also appears to be required for their proliferation, but in that case this contact seems not to be HA-dependent (Sugimoto *et al.,* 1994).

1.3 The aims of the project and system used

The basis for this work was the observation that HA treatment of LTBMC stimulates the production of progenitor cells and mature cells. HA treatment induces signalling that leads to the production of IL-1β and IL-6 cytokines. To gain a mechanistic understanding how HA stimulates cytokine production, we wanted to establish a simple system, composed of cell lines, which mimics at least some steps of LTBMC. As a substitute for stromal cells we took the stromal cell line MS-5, which supports steps of hematopoiesis in several

systems, including some progenitor cell lines. As progenitor cells we used the erythroleukaemic progenitor cell line TF-1, which requires for its growth the cytokines GM-CSF or IL-3 cytokines and responds also to IL-6. We aimed to investigate the responsiveness of MS-5 to HA, study the cytokine production in these cells and eventually elucidate the mechanism of IL-6 regulation. This response of MS-5 cells to HA should be compared with the ability of MS-5 cells to support the progenitor cell line TF-1.

2 Materials and Methods

2.1 Materials

All general chemicals were supplied from Merck (Darmstadt), Carl Roth GmbH & Co (Karlsruhe), Sigma Chemie GmbH (Diesenhofen) and were of highest purity grade. The HA (from rooster comb) and other polysaccharides were dissolved in H_2O and then incubated for 10 min at 95¡C.

2.1.1 Mice

C57bl/6 mice were provided from Jackson Laboratories (USA), kept under specific pathogen-free conditions and used for experimental passage at the age of 8-12 weeks.

2.1.2 Cell lines and mediums

All cells were maintained in a 37°C incubator in a humid atmosphere with 5% CO_2. Cell stocks were maintained at -80°C and in liquid nitrogen. Trypsin was obtained from Difco Laboratories (Detroit, USA) and diluted to 0,25% in 15 mM sodium citrate, 134 mM potassium chloride. EDTA (Merck, Darmstadt) was used as a 5 mM solution in PBS.

The **MS-5** cell line is bone marrow derived stromal cell line (Itoh *et al.*, 1989). MS-5 cells were maintained in Alpha medium containing 10% FCS and 2mM Glutamin.

The **TF-1** cell line is a $CD34^+$ human erythro-myeloid progenitor cell line. TF-1 cells depend for the growth on either GM-CSF, IL-3, IL-6 and for the differentiation on erythropoietin (Kitamura *et al.*, 1989a; Kitamura *et al.*, 1989b; Kitamura *et al.*, 1991). TF-1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 500 U/ml of human recombinant GM-CSF.

The **HA9** cell line (a kind gift of D. Naor) is a clone from a mouse T-cell lymphoma LB cell line which is constitutively binding hyaluronic acid. HA9 cells were maintained in RPMI 1640 medium containing 10% FCS, 10 mM Hepes (pH=7,4), 1% non-essential amino acids, 1 mM sodium pyruvate, 1mg/ml insulin, 0,05 mM β-Mercaptoethanol.

2.1.3 Plasmids and constructs.

PGL2 vector (Promega); p3xκB-§Glo-TATA-luc (3xNF-κB) and §Glo-TATA-luc (vector control) were kindly provided by Dr.Weih; Raf-C4 dominant-negative and Raf-wt constructs (Bruder *et al.,* 1992); p50pcDNA1 (Invitrogen); p65/RelApcDNA1 (Invitrogen)

IL-6 promoter deletion and point-mutated constructs, containing the luciferase reporter gene, were the kind gift from Dr.Vellenga:

Name	**Description**
1)pIL6luc(-602)	- 602 bp upstream of IL-6 transcription start
2)pIL6luc(-298)	- 298 bp upstream of IL-6 transcription start
3)pIL6luc(-235)	- 235 bp upstream of IL-6 transcription start
4)pIL6luc(-169)	- 169 bp upstream of IL-6 transcription start
5)pIL6luc(-122)	- 122 bp upstream of IL-6 transcription start
6)pIL6luc(-60)	- 60 bp upstream of IL-6 transcription start
7)pIL6lucNF-κBm	- 602 bp upstream of IL-6 transcription start, - NF-κB mutated site.
8)pIL6lucNF-IL-6m	- 602 bp upstream of IL-6 transcription start, - NF-IL-6 mutated site.

2.1.4 Radiochemicals.

[^{3}H] Thymidin	Amersham (Braunschweig)
[p32] dCTP	Amersham (Braunschweig)

2.1.5 Primers

All primers were produced and HPLC purified by MWG biotech (Ebersberg) or Birsner&Grob Biotech GmbH.

2.1.5.1 Primers used for CD44 exon-specific RT-PCR analysis

C13	5'-AAG ACA TCG ATG CCT CAA AC-3'	constant region, oligo3'
C2A	5'-GGC ACT ACA CCC CAA TCT TC-3'	constant region, oligo5'
pV2	5'-GAT GAC TAC CCC TGA AAC AC-3'	variant region, oligo5'
pV3	5'-ACG GAG TCA AAT ACC AAC CC-3'	variant region, oligo5'
pV4	5'-TGC AAC TAC TCC ATG GGT TT-3	variant region, oligo5'
pV5	5'-TAT AGA CAG AAA CAG CAC CA-3'	variant region, oligo5'
pV6	5'-TGG GCA GAT CCT AAT AGC AC-3'	variant region ,oligo5'
pV7	5'-CTG CCT CAG CCC ACA ACA AC-3'	variant region, oligo5'
pV8	5'-CCA GTC ATA GTA CAA CCC TT-3'	variant region, oligo5'
pV9	5'-CAG AAC TTC TCT ACA TTA CC-3'	variant region, oligo5'
pV10	5'-GGT CGA AGA AGA GGT GGA AG-3'	variant region, oligo5'
GAPDH5'	5'-GTT CGA CAG TCA GCC GCA TCT-3'	control GAPDH
GAPDH3'	-5-TTC TCC ATG GTG GTG AAG ACG-3'	control GAPDH

2.1.5.2 Primers used for cloning the IL-6 promoter construct pIL6luk(1,3)

IL65p 5'-GAG CTC GAG GGA TCC TGA GAG TGT GT-3'

IL63p 5'-CTC AGA TCT AGC GGT TTC TGG AAT TGA-3'

2.1.6 Antibodies

2.1.6.1 Primary Antibodies

Name	Description	Supplier/Reference
KM81	anti-mouse CD44 rat mAbs HA-binding domain	ATCC TIB 241
KM81(Fab')$_2$		Eurogentec Bel S.A.
IM7	anti-mouse/human pan CD44 rat mAbs IgG2b	PharMingen
ERK-1/2	rabbit polyAbs	Santa Cruz, USA
p38	rabbit polyAbs	Santa Cruz, USA
SAPK/JNK	rabbit polyAbs	New England Biolabs
Phospho ERK-1/2	rabbitpolyAbs	New England Biolabs
Phospho p38	rabbit polyAbs	New England Biolabs
Phospho SAPK/JNK	rabbit polyAbs	New England Biolabs

Name	Description	Supplier/Reference
VFF4	anti-human CD44v6 murine mAbs IgG2b	Bender+Co Ges GmbH
VFF8	anti-human CD44v5 murine mAbs IgG1	Bender+Co Ges GmbH
VFF14	anti-human CD44v10 murine mAbs IgG1	Bender+Co Ges GmbH
VFF16	anti-human CD44v10 murine mAbs IgG1	Bender+Co Ges GmbH
VFF17	anti-human CD44v7-8 murine mAbs IgG2b	Bender+Co Ges GmbH
VFF18	anti-human CD44v6 murine mAbs IgG1	Bender+Co Ges GmbH
CD44-FITC	anti- mouse CD44	PharMingen
CD44- PE	anti- mouse CD44	PharMingen

2.1.6.2 Secondary Antibodies.

Name	Description	Supplier/Reference
Rabbit HRP	goat IgG	Dako, Hamburg
Mouse FITC	goat IgG	Pharmingen
Mouse PE	rabbit IgG	Dako, Hamburg
Rat FITC	goat IgG	Pharmingen
Rat PE	goat Ig(H+L)	Pharmingen

2.2 Methods

A number of protocols and prescriptions for commonly used buffers were taken from the Laboratory Manual of Maniatis et al. (Maniatis *et al.*, 1989) and from the Current Protocols in Molecular Biology (Ausubel *et al.*, 1989).

2.2.1 Restriction endonuclease digestion of DNA

DNA was digested at a final concentration of 1-5 μg/μl. Digestion was performed with 3-5 units of enzyme per 1 μg DNA in the buffer recommended by suppliers.The reaction was maintained at 37°C for 2 hours till O/N and terminated by phenol/chloroform extraction and ethanol precipiation.The digestion products were analysed by agarose gel electrophoresis.

2.2.2 Phenol/chlorophorm extraction

To remove contaminating proteins from nucleic acids, a mixture of Tris buffered phenol, chloroform and isopropanol (2-propanol) at a ratio 25:24:1 was added and vortexed in. The phases were separated by centrifugation at 14000 rpm for 5 min. The upper phase, containing the nucleic acids was transferred into a new tube and used for a next round of extraction with chloroform/isopropanol (24:1).

2.2.3 Isolation of DNA fragments from agarose gel

a) Direct method of DNA isolation from agarose gel

The necessary DNA band was cut out from the gel with a scalpel under long wave UV light. The gel strip, containing the DNA, was placed into a pierced 0,5 ml reaction tube containing glass wool, which in turn was placed inside a 1,5 ml Eppendorf tube and centrifuged at 12000 rpm for 30 min. The DNA was collected in the reaction tube, leaving the agarose caught in the glass wool. The DNA was then extracted with phenol chloroform and precipitated with ethanol.

b) EasyPure Kit (Biozyme) DNA isolation

The necessary DNA band was cut out and the gel piece was added to 3 volumes (w/v) of Salt Buffer and incubated for 15 min at 65¡C. After adding binding resin the mixture was incubated for 5 min at RT. The resin with bound DNA was washed twice with ethanol and air dried. The DNA was diluted with bidist. H_20 or TE buffer (10 mM Tris-HCl, pH=8,0; 1 mM EDTA).

2.2.4 Precipitation of nucleic acid

The precipitation of DNA and RNA was done by adjusting the final salt concentration of the reaction mixture to 300 mM using 3M Na-acetate. The precipitate was pelleted by centrifugation at 14000 rpm at 12^0C for 30 min, washed twice with 75% ethanol and allowed to air dry for 15 minutes.

2.2.5 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their OD (optical density) at 260 and 280 nm. One OD_{260nm} is equvalent to 50 µg/ml of double strained DNA and 40 µg/ml of RNA. The OD_{280nm} is an indicator of the purity of the nucleic acid and should have approximatly 50% of the value of OD_{260nm}.

2.2.6 Size separation of nucleic acid by agarose gel electrophoresis

The required amount of agarose was dissolved in TAE buffer (20 mM Tris-HCl, 10 mM acetic acid, 1 mM EDTA, pH=8,3) and boiled. The ethidium bromide was added until the final concentration was 0,3 µg/ml. The gel was poured into a horizontal (13,5x8 cm) chamber containing the appropriate number and size of the teeth to make loading slots. When set, the gel was covered with TAE buffer and DNA samples were loaded into the wells using loading buffer (10 mM EDTA, 10% v/v glycerol, 0,1% w/v SDS, 0,02% bromphenol blue). The migration of DNA was visualised by UV ligh transilluminator.

2.2.7 Preparation of competent cells

A single colony of E.coli DH5α was transferred into 3 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and incubated O/N at 37°C with shaking (220 rpm). 4ml of the O/N culture were taken to innoculate 400 ml of LB medium, grown to an OD_{590nm} of 0,375 and incubated on ice for 15 min. The bacteria were pelleted by centrifugation at 3600 g for 10 min at 4°C, pellet was resuspended in 20 ml of ice-cold 0,1 M $CaCl_2$ and incubated on ice for 15 min. The process was repeated and the pellet was finally resuspended in 2 ml of ice-cold $CaCl_2$ with 10% glycerol.
The bacteria were kept in 80µl aliquots at -80¡C.

2.2.8 DNA ligation

Ligation reaction was usually performed in a volume of 20 µl. Usually, 100-200 ng of DNA fragment were mixed with 300-500 ng of vector DNA and with 2 µl of 10x Ligase buffer. 1 µl of T4 ligase was added and the reaction was performed O/N at 16¡C.

2.2.9 Transformation of competent bacteria

The transformation was done by electroporation using the Gene Pulser from Biorad. Usually, 1 µl of a ligation mixture was added to 40 µl of electrocompetent cells and incubated on ice for 1 min. Cells were transferred

into pre-cooled electroporation cuvette (0,1 cm, BioRad) and pulsed at 1,8 kV. Immediately after pulsing, the cells were supplemented with 1 ml SOC medium (20 g/l Bacto trypton, 5 g/l Yeast extract, 0,5 g/l NaCl, 2,5 mM KCl, 10mM $MgCl_2$, 10 mM $MgSO_4$, 2mM Glucose) incubated at 37°C for 45 min and plated (100 µl of the cell mixture) on LB- agar plates (1% Bacto trypton, 0,5% Yeasr extract, 1% NaCl, 1% Agar) with the appropriate antibiotic.

2.2.10 Small scale plasmid preparation (Miniprep)

3 ml of LB mediom containing the appropriate antibiotic were inoculated with a single bacterial colony and incubated O/N at 37°C. O/N culture was centrifuged for 5 min at 14000 rpm; the pellet was resuspended in 150 µl of GTE solution (50 mM glucose, 25 mM Tris-HCl, pH=8,0) and incubated on ice for 5 min. After this, 200 µl of the lysis buffer (0,2 M NaOH and 1% w/v SDS) were added, briefly vortexed and incubated again on ice for 5 min; 150 µl of 3 M Na-acetate, pH=5,2 were added, the contents mixed by inversion of the tube and incubated on ice for 5 min. After 15 min of centrifugation at 14000 rpm, the supernatant was transferred into a tube containing an equal volume of phenol/chloroform, vortexed and centrifuged for 5 min at 14000 rpm. The aqueous phase containing the DNA was removed to a fresh tube and precipitated with 1ml of pre-cooled ethanol and 55 µl of 3M NaAc. The DNA pellet was air dried and resuspended in 20 µl of TE buffer (10 mM Tris-HCl, pH=8,0; 1 mM EDTA).

2.2.11 Large scale plasmid preparation (Maxiprep)

The large scale plasmid preparation was perfomed using the Qiagen Kit. A single bacterial colony was picked and transferred into 5 ml of LB medium with the appropriate antibiotic, and incubated for several hours at 37°C. 1 ml of this medium was inoculated into 500 ml of LB medium with antibiotic and incubated O/N at 37°C. The bacteria were pelleted by centrifugation at 6000 rpm at 4°C for 15 min and resuspended in 10 ml of P1 buffer (50 mM Tris-HCl; 10 mM EDTA; 100 µg/ml RNase A). Bacteria were lysed by addition of P2 buffer (200 mM NaOH, 1 % w/v SDS), carefully mixed and incubated for 5 min at RT. 10 ml of P3 buffer (2,55 M KAc, pH=4,8) were gently mixed in

and after 20 min of incubation on ice the precipitated material was centrifuged down at 12000 rpm, at 4°C for 30 min. The supernatant was transferred to a Qiagen-tip 500 column, pre-equilibrated with QBT solution (750 mM NaCl, 50 mM MOPS, 15% v/v), and allowed to drip through.
The bound DNA was washed twice with 30 ml of QC solution (1M NaCl, 50 mM MOPS, 15% v/v ethanol, pH=7,0) and eluted with 5 ml of QF buffer (1,25 M NaCl, 50 mM MOPS, 15% v/v ethanol, pH=8,2) and precipitated with 12 ml of isopropanol. After a washing step with 70% ethanol the DNA pellet was resuspended in 200 µl TE. The amount of obtained DNA was determined by measuring its OD (optical density) at 260 nm.

2.2.12 Radioactive labelling of DNA probes

Radioactive labelling of DNA probes was done with the ReadyPrime kit (Amersham Life Science) using [p32]-dCTP (Amersham Buchler GmbH, Braunschweig, 370 Mbq/ml, 10 mCi/ml) according to the manufacturers protocol. Unincorporated nucleotides were removed from labelled DNA using Chromospin+STE-100 columns (Clontex Laboratories, Inc.).

2.2.13 Polymerase Chain Reaction (PCR)

Usually the PCR was performed in a total volume of 10-100 µl; the PCR mixture consisted of 1 pmol of 3' and 5' primer(s), 250 µM of dNTP mix (10 mM of each: dATP, dCTP, dGTP, dTTP), 1x appropriate suppliers buffer with a defined concentration of $MgCl_2$, 1-4 U of Taq polymerase (prepared by J.Moll) and variable amounts of DNA. The reaction was carried out in the commercially available PCR machine (Peltier Thermal Cycler, MJ Research). Specific cycling parameters were used depending on the application.
For cloning the 1,3 kb part of the IL-6 promoter following parameters were used:

94°C	1min	1 cycle
94°C	1min	
56°C	1min	30 cycles
72°C	1min	

The PCR products were analysed using gel electrophoresis.

2.2.14 CD-44 exon specific RT-PCR analysis.

Poly A^+RNA was prepared from the MS-5 cells and used for single strain cDNA synthesis.

1-2 µg of poly A^+RNA were mixed with 500 ng of oligo$(dT)_{12-18}$ primer in a total volume of 12 µl, heated to 70°C for 10 min and rapidly chilled on ice. The mixture was supplemented with 4 µl of 5x first strand reaction buffer, 2 µl of 0,1 M DTT and 1 µl dNTP mix (10 mM of each: dATP, dCTP, dGTP, dTTP). 1 µl of SuperScript reverse transcriptase (Gibco BRL) was added just before incubation and the reaction was performed at 42° for 1 hour. The reaction was terminated by heating the mixture to 94°C for 3 min and placing the tube on ice. This first strand reaction was stored at -20°C and used later for RT-PCR reaction.

RT-PCR reactions were performed as described for normal PCR with the exeption that first strand cDNA was used as template DNA.

The following PCR parameters were used for CD44 exon-specific RT-PCR:

94°C	10 min	1 cycles
95°C	30 sec	
50°C	1 min	35 cycles
72°C	2 min	

The PCR was performed in total volume 100 µl.

The mixture consisted of:

2,5 µl	cDNA,
2 µl	oligo 3'
2 µl	oligo 5'
10 µl	10xbuffer
2,5 µl	dNTPs
8 µl	25mM $MgCl_2$
0,5 µl	Taq polymerase
72,5 µl	bidest. H_20

C2A primer located in the 3' constant region of CD44 was used as oligo 5'. C13 primer located in the 5'constant region of CD44 and individual variant exon-specific primers (pV1-pV10) was used as oligo3'. GAPDH5' and GAPDH3' primers were used for the positive control. The PCR products were analysed by agarose gel electrophoresis.

2.2.15 DNA isolation

For the isolation of total genomic DNA, 750 µl of proteinase K buffer (50 mM Tris-HCl, pH=8,0; 100 mM EDTA; 100 mM NaCl; 1% SDS w/v; 0,5 mg/ml proteinase K) were added to the cell pellet (or to the 2cm piece of mouse tail) and incubated O/N at 55°C. The resulting suspension was supplemented with 250 µl of 6M NaCl and mixed for 5 min with an Eppendorf mixer. Contaminating material was pelleted by 5 min centrifugation at 14000 rpm and 750 µl of the supernatant (without top phase and pellet) were transferred into the new tube. The DNA was precipitated with an equal volume of isopropanol, washed with 75% ethanol and incubated with 300 µl of TE buffer for 2 hours at 37°C. The amount and quality of the obtained DNA were analysed on an agarose gel and by measuring its OD at 260 nm.

2.2.16 Isolation of total RNA

TRI REAGENT (Sigma) was used for the isolation of total RNA and simultanious isolation of DNA and proteins. Cells were directly lysed with 1 ml of TRI-REAGENT on 9,4 cm culture dishes. The aqueous phase was transferred to a fresh tube and 0,2 ml of chlorophorm and 0,5 ml of isopropanol was added. Samples were incubated for 5 -10 min at RT and centrifuged at 14000 rpm for 10 min at 4°C. The pellet was washed with 1ml of 75% ethanol, air dried and dissolved in bidist. H_20. The amount of obtained RNA was determined by measuring its OD at 260 nm.

2.2.17 Isolation of Poly A^+ RNA

Cells were washed twice with PBS and harvested from 15 cm dishes using 10 ml of STE-SDS buffer (100 mM NaCl, 20 mM Tris, pH=7,4; 10 mM EDTA;

0,5% w/v SDS) containing 300 mg/ml proteinase K. The cell mixture was homogenised with a ultraturax and incubated at 55°C for 30 min. After the incubation, the solution was supplemented with 1ml of 5 M NaCl and 100 µg Oligo-dT-cellulose. The suspension was rotated O/N at RT to permit binding of polyA$^+$ RNA. Oligo-dT-cellulose was centrifuged for 3 min at 2000 rpm at 12°C and washed 3 times with 10 ml HSB buffer (300 mM NaCl; 10 mM Tris, pH=7,4; 5 mM EDTA; 0,1% w/v SDS) and once with distilled H_2O.
In order to elute DNA, Oligo-dT-cellulose was washed 3 times with H_2O; After each centrifugation the supernatant was collected in fresh pre-cooled tube. Once completed, RNA was precipitated by addition 2,5 volumes of ethanol, 200 µl NaAc and 6 µl tRNA as a carrier and incubated at -20°C O/N. RNA was pelleted by 30 min centrifugation at 10500 rpm in a Beckman Swing out rotor at 4°C, washed once with 75% ethanol and resuspended in bidest. H_2O. The amount of the RNA obtained was determined by measuring its OD at 260 nm.

2.2.18 Northern blot hybridisation

Usually 5 µl of RNA solution (1µg/µl) were mixed with 15 µl of formaldehyde denaturing buffer (50 µl 10xMOPS, 87,5 µl 37% v/v formaldehyde, 250 µl formamide, 3 µg/ml ethidium bromide) and denaturated at 65°C for 15 min. Samples were suplemented with 2 µl of 10x loading buffer (10 µl 0,5 M EDTA, 5ml 50 % glycerol, 0,1 % w/v bromphenol blue)
The 100 ml of RNA-gel- mix consisted of 1g agarose, 10ml 10xMops(41,85g MOPS, 6,8g NaOAc x 3 H_2O, 20 ml 0,5 M Na_2EDTA in 1 L H_2O, pH=7,0), 3,5 ml formaldehyde, 85 ml bidest. H_2O). The gel was poured into a chamber with the comb containing the appropriate number and size of the teeth to make loading slots. When set, the gel was covered with 1xMOPS running buffer and the RNA samples were loaded into the wells. The migration of RNA was visualised by a UV light transilluminator and photographed. The gel was blotted O/N on a Hybond N$^+$ membrane with 20 x SSC. The RNA was covalently crosslinked to the active OH groups in the membrane by a UV stratalinker 2400.
Hybridisation was performed in Church buffer (0,25 M $NaPO_4$, 7% w/v SDS, 1 mM EDTA pH=8,0) O/N at 65°C.

Filter was washed 2 times with Church buffer and once with 1% SDS/1x SSC at 65°C. The filter was sealed in a plastig bag and exposed to an Amersham Hyperfilm or to Phosphorimager screen.

2.2.19 Transient transfection

The transient transfections were performed by electroporation using Electroporation dense pulse.
One day before use the cells were replated a in 9,4 cm culture dishes and allowed to reach 70% confluence. After trypsinisation, cells were resuspended in 400 µl of medium containing 10% FCS, mixed with 2-10 µg DNA and transferred to the pre-cooled 0,4 ml cuvettes; they were pulsed at 270V, 250µF, immediately supplemented with 400 µl of FCS and plated in 10 cm culture dishes. The transformation efficiency was monitored by co-transfection with GFP (green fluorescence protein) and checked 24-48 hours after electroporation by FACS or fluorescent microscopy.

2.2.20 Measurement of luciferase activity

The cells used for assay were growing in 9,4 cm dishes. After removing the medium, the cells were washed twice with PBS without Ca^{2+} and Mg^{2+} and incubated with 400 µl of lysis buffer (0,1 M Tris acetate, pH=7,5, 2 mM EDTA, 1% Triton X-100 v/v) on ice for 20 min (with occasional rocking). The cell lysates were collected with the rubber policemen into precooled tubes, and cleared by 5 min centrifugation at 12000 rpm. To determine the luciferase activity, 100 µl of the lysate were transferred to a reading tube. The measurement was done by a Luminator (Berthold, Wildbad) with autoinjection of 350 µl of assay buffer (1 mM DTT, 1 mM ATP in glycylglycine buffer (25 mM GlyGly, 15 mM $MgSO_4$ and 4 mM EGTA) and 100 µl of luciferin assay solution (1mM luciferin stock solution (0,28 mg/ml) in glycylglycine buffer [1:5]).

2.2.21 Transwell assay

Adherent cells were cultured in 6-well culture plates until 70% confluence. To avoid the direct contact between the two cell lines, the suspended cells were

added to the same plates in the presence of transwell filters (40 µm). In this case, cells were still cultured in the same medium volume but separated from direct interaction. The supernatants were investigated for the amount of cytokines using an ELISA kits.

2.2.22 LTBMC

Myeloid LTBMCs (Long term bone marrow culture) were established as described (Whitlock et al., 1982). In brief, femurs and tibias were removed from mice, which were killed by cervical dislocation. Bone marrow cells were flushed out of the bones, using Dulbecco medium containing 2 % FCS. The isolated bone marrow cells were washed twice, counted and cultured in DMEM, supplemented with 20 % horse serum (Linaris) and Hydrocortisone (10^{-6} M; Sigma) in 6 or 24 well plates at 33°C in a humid atmosphere containing 5% CO_2.

2.2.23 ELISA

Cells were stimulated with HA (100 µg/ml) or other reagents. The supernatant samples were collected and kept frozen at -80°C. The frozen samples were checked by ELISA (Enzyme-linked immunosorbent assay)-kits (Biozol) for the presence of different cytokines according to the manufacturer instructions.

2.2.24 Immunohistochemistry (staining of actin cytoskeleton)

Cells were washed twice with PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6,9), fixed with 2,5% glutaraldehyde (Serva) in stabilising buffer (20 mM Hepes, 5 mM EGTA, 2 mM PMSF, 1 mM $MgSO_4$, 1g/l Na-Tosyl- L-argininmethylester, 20% v/v Glycerin, pH=6,8) at 37°C for 15 min. Thereafter cells were treated with 50-100 µl staining solution (3,3 mM phalloidine -rhodamine in methanol 1:10 in PHEM-buffer) at 37°C for 2 hours. The staining was visualised by fluorescence microscopy.

2.2.25 Flow cytometry

To investigate the expression of molecules on the cell surface, cells were harvested, pelleted and dissolved in FACS buffer (PBS, 3% FCS). After 15 min of preincubation, $5x10^5$ cells per probe were incubated with 10 µg/ml of primary antibodies in FACS buffer (30 min, on ice). After two washing steps with PBS, probes were incubated with appropriate FITC-, or PE- labeled antibodies and washed twise again. To detect HA-binding, cells were incubated with FITC-labeled HA instead of primary and secondary antibodies.
The fluorescence was analysed by FACS-Star plus flow cytometer (Becton Dickinson).

2.2.26 [^{3}H] Thymidin incorporation

The cells were plated in 96-well plates (10^5/per well), allowed to grow O/N and treated with the reagents of interest. [^{3}H] Thymidin was added until a final concentration of 1 µCi/ml. After 6 hours the cells were harvested by a TOMTEC Harvester (Wallac ADL GmbH) and amount of incorporated radioactivity was analysed by 1450 Microbeta liquid scintillation and luminiscence counter (Wallac ADL GmbH).
For proliferation-analysis of TF-1 cells on a MS-5 feeder layer, the MS-5 cells were allowed to grow until confluence in 6-well plates and were irradiated with 900 rad by γ-ray. TF-1 cells (10^5) were added directly on MS-5 stroma. After 12-24 hours of co-culture, the cells were pulsed with [^{3}H] Thymidin for 6 hours, harvested and analysed as discribed before.

2.2.27 Immunoblot analysis of proteins (Western blot)

a) Cell lysates

Cells were grown to confluence, treated with appropriate reagents and harvested by scraping with a rubber policeman in the presence of sample buffer (125 mM Tris pH=6,8, 2% w/v SDS, 0,02% bromphenol blue), containing 10% glycerol for nonreducing, and 100 mM Dithiothreitol (DTT) for reducing conditions. Samples were boiled for five minutes, sonicated for 30 seconds and kept frozen at -80^{0}C.

b) SDS- PAGE

Proteins were separated electrophoretically on the basis of molecular weight using the method of Laemmli.

The running gel mixture was poured between glass plates separated with special spacers until two thirds full, overlaid with ethanol and allowed to polymerase.The stacking gel mixture was poured on top of the running gel. The sample wells were formed by appropriate combs placed in the stacking gel. The gel was fixed into a vertical running chamber filled with electrophoresis buffer (25 mM Tris, 192 mM glycine, 0,1 % w/v SDS).

Running gel mix (25 ml):

30% acrylamid-bisacrylamide	8,325 ml
1,5 M Tris pH=8,8	6,25 ml
20% SDS	0,125 ml
H_2O	10,05 ml

+250 µl Ammonium persulfate (10% w/v)

+15 µl TEMED

stacking gel mix (20 ml)

30% acrylamid-bisacrylamide	3,4 ml
0,5 M Tris pH=6,8	5,00 ml
20% SDS	0,1 ml
H_2O	11,3 ml

+200 µl Ammonium persulfate (10% w/v)

+20 µl TEMED

Samples were run into the stacking gel at 70V and then at 30V overnight or 140V during the day.

c) Protein staining with Coomassie Brilliant Blue

In order to visualise proteins in SDS-PAGE, the gel was incubated for 1-2 h in staining solution (50% methanol, 7,5% acetic acid, 0,2% w/v Coomassie brilliant blue R250) and destained for several hours in a destaining solution (50% methanol; 7,5% acetic acid) which was changed until the gel background is clear. Once completed, the gel was dried on What 3MM paper.

d) Western blotting

Proteins resolved by SDS-Page were transferred to Immunobilon-PVDF membrane (Milipore, Bedford, UK) using BioRad Transfer chambers containing blotting buffer (20 mM Tris, 192 mM glycine, 0,1% w/v SDS, 20% v/v methanol). According to the size of the protein and the current applied transfer time varied from 4-6 hours to O/N.

e) Western blot probing

The membrane was briefly washed in PBS, preincubated for 1 hour in blocking buffer (PBS+5% w/v BSA, 0,1% v/v Tween) and incubated with the primary antibody for 1h to O/N (according the manufacturer instructions for the antibody) at 4°C. After 3 washing steps with PBS + 0,1% v/v Tween, the membrane was incubated another hour with the secondary antibodies at RT and washed 3 times again with PBC. The proteins were visualised by ECL reaction (Amersham) and exposed to the ECL- Hyperfilm (Amersham).

2.2.28 CPC precipitation

a) Biotinylation of cell surface proteins

Cells were washed three times with ice-cold PBS (without Ca^{2+}, Mg^{2+}) and re-suspended in freshly prepared 1 mg/ml NHC-biotin/PBS solution (NHS-LC-Biotin, Pierce) at a concentration $5x10^5$ cells/ml. The cell mixture was incubated on ice for 1 hour with additional rocking. After incubation, cells were washed 3 times with PBS containing 200 mM of glycine. The biotinylated cells were used for CPC precipitation.

b) Cell preparation.

Previously biotinylated cells or confluent cells of a 15 cm dish, washed three times with PBS were used for the preparation of cell lysate. They were lysed with 1 ml PBS/0,5% NP40, supplemented with PMSF (1 mM final concentration), incubated on ice for 30 min. and centifuged at 14000 rpm, 10 min in order to pellet insoluble material; the supernatant was taken as the cell lysate. 100 µl of cell lysate were mixed with 350 µl of reducing sample buffer and 50 µl 1M DTT and stored as a positive control.

c) Precipitation

Aliquots (100 µl) of the cell lysates were used for CPC (Cetylpyridinium chloride (Sigma chemical co, USA)) precipitation.

Glycosaminoglycans were dissolved in water to a final concentration of 1mg/ml. 50 µl of glycosaminoglycan solution were mixed with 100 µl of cell lysate and incubated at room temperature for 1 hour.Once completed, 350 µl of a 1.4% CPC solution were added to the mixture and incubated for another hour at RT. Precipitate was pelleted by 10 min centrifugation in a swing out rotor and washed 3 times with 1ml of 1%CPC/30mM NaCl and disolved in 50 µl of reducing sample buffer. Samples were shaked for 30-60 minutes to dissolve the pellet completely, boiled and loaded on a SDS-PAGE gel. SDS-gel was bloted as for a Western blot. In the case of biotinylated cells, the blot was probed with HRP coupled to streptavidin (Dianova) and analysed by ECL reaction. For CD44 detection the procedure was done at non-reducing conditions and the blot was probed with IM7 antibodies.

3 Results

3.1 Part 1: HA mediated cytokine induction in MS-5 cells

The starting point of this project was the observation that HA, a component of the bone marrow environment, can significantly enhance hematopoiesis and plays a regulatory role in this process (Khaldoyanidi *et al.*, 1999).
Exogeneously added HA increases the number of myeloid and lymphoid non-adherent cells in LTBMC and also the total amount of clonogenic cells. It was shown that HA acts on hematopoiesis via stromal cells, which respond to HA by upregulation of IL-6 and IL-1β cytokines. The enhancing HA effect on LTBMC was due to IL-6 cytokine release from bone marrow macrophage stromal cells.

3.1.1 Stromal cell lines can support hematopoietic progenitors

The hematopoietic potential of LTBMC can be partially simulated by a system consisting of progenitor cells and hematopoietic stromal cell lines (Dorshkind, 1990). To test whether in such a system HA would also stimulate cytokine production similarly to the effect in LTBMC we used the bone marrow stromal cell line MS-5 and the $CD34^+$ progenitor cell line TF-1.
At first, we tested whether MS-5 cells can support the survival and the proliferation of TF-1 cells. For this purpose, MS-5 cells were grown up to confluence and were subsequently irradiated by γ-ray (9OO rad). TF-1 cells were then seeded on the MS-5 layer and their proliferation was examined at different times by the [^{3}H] thymidin incorporation method. TF-1 cells were also grown in the presence of recombinant GM-CSF (positive control) in the medium and without cytokine addition (negative control). At each time point for the last 6 hours thymidin was added. Afterwards cells were harvested and incorporated radioactivity was counted.
After 24 hours we could observe a significant difference in proliferation between co-culture of TF-1 cells with MS-5 cells and TF-1 cultures without cytokine addition, which became even more pronounced after 36 hours (Fig.5).

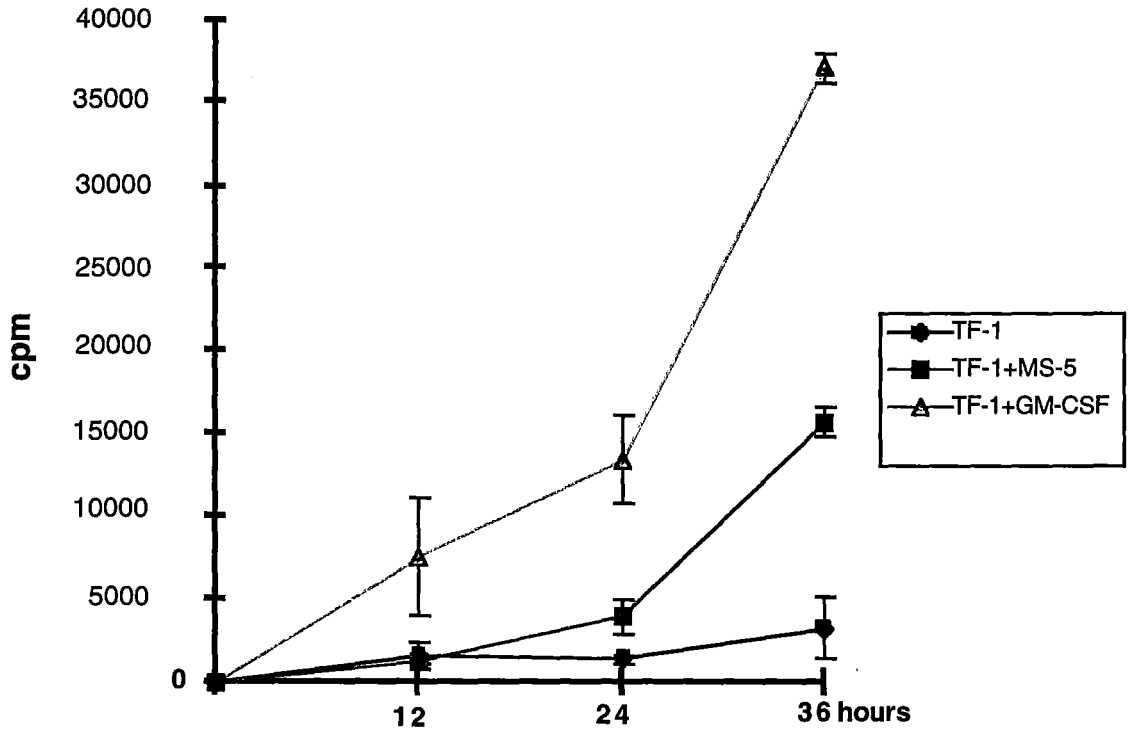


Figure 5. MS-5 feeder layer supports the survival and the proliferation of TF-1 cells. *MS-5 cells were grown up to confluence and irradiated with 900 rad by γ-irradiation. TF-1 cells (10^5) were co-cultured with the MS-5 feeder layer. The proliferation of TF-1 cells was examined with the (^3H) thymidin incorporation assay. TF-1 cells alone and supplemented with rGM-CSF were used as negative and positive controls. SD was calculated from results obtained from triplicates.*

The TF-1 cells in the absence of cytokines or MS-5 cells did not proliferate. TF-1 cells in the presence of recombinant GM-CSF (rGM-CSF) showed proliferation as did TF-1 cells grown on MS-5 feeder layer. Thus, we conclude that the MS-5 cell line can support the growth of TF-1 cells. This result suggests that MS-5 cells produce a basic amount of one of the factors, which could support the growth of the TF-1 cell line. Indeed it was previously described that MS-5 cells produce GM-CSF and IL-6. Thus the MS-5 clone had the desired property to support hematopoietic progenitors, an absolute requirement for our further studies.

3.1.2 MS-5 cells can bind FITC- labelled HA on their surface

To study the influence of HA on MS-5 cells we examined binding of HA to the MS-5 cell line. Cells were pre-treated with hyaluronidase (HA ase) and then incubated with FITC- labelled HA. Bound HA was measured by FACS-Star plus flow cytometer. The method demonstrated HA binding to MS-5 cells (Fig.6).

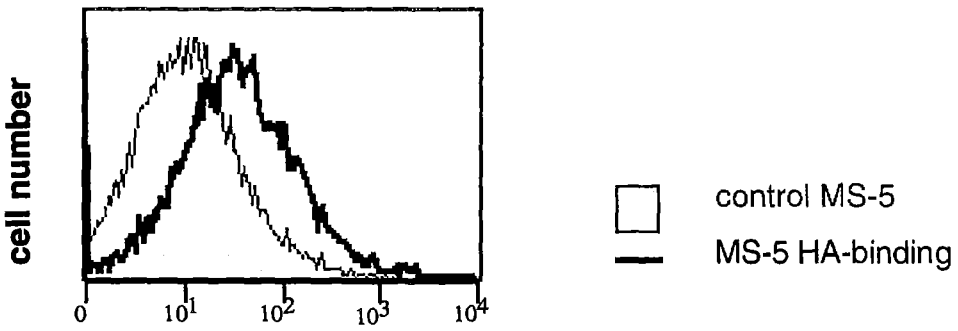


Figure 6. MS-5 cells bind HA (FACS staining with FITC-HA). *10^5 cells were stained with FITC labelled HA. The fluorescence was analysed on a FACS-Star plus flow cytometer.*

3.1.3 HA specifically stimulates cytokine production by MS-5 cells

We tested whether MS-5 cells respond to HA treatment by the induction of cytokines similarly to bone marrow derived macrophages (BMDM) in LTBMC. For this purpose MS-5 cells were grown up to 70 % confluency and treated with HA. The production of cytokines was measured using ELISA kits. HA treatment increased the production of IL-6, GM-CSF and IL-4 cytokines, whereas IL-3 and IL-1β production was not influenced (Fig.7). To further characterise our system we examined the time course and concentration dependence of cytokine production induced by HA (Fig.8). The first significant increase in cytokine production was seen 6 hours after the addition of HA. The maximal increase was reached after 24 hours. The specificity of HA action was demonstrated by the fact that a treatment with chondroitin sulfate (CS), another glycosaminoglycan, had no effect on MS-5 cells (Fig.9).

Our results indicate that the MS-5 cell line responds to HA treatment by upregulation of cytokines relevant for hematopoiesis, particularly of IL-6. Although other cytokines are released by MS-5 cells than the ones from BMDM, we believe that this difference reflects the reaction of particular stromal cell compartments. Therefore, the MS-5 cell line could be an excellent model to assess the mechanism of cytokine activation upon HA treatment.

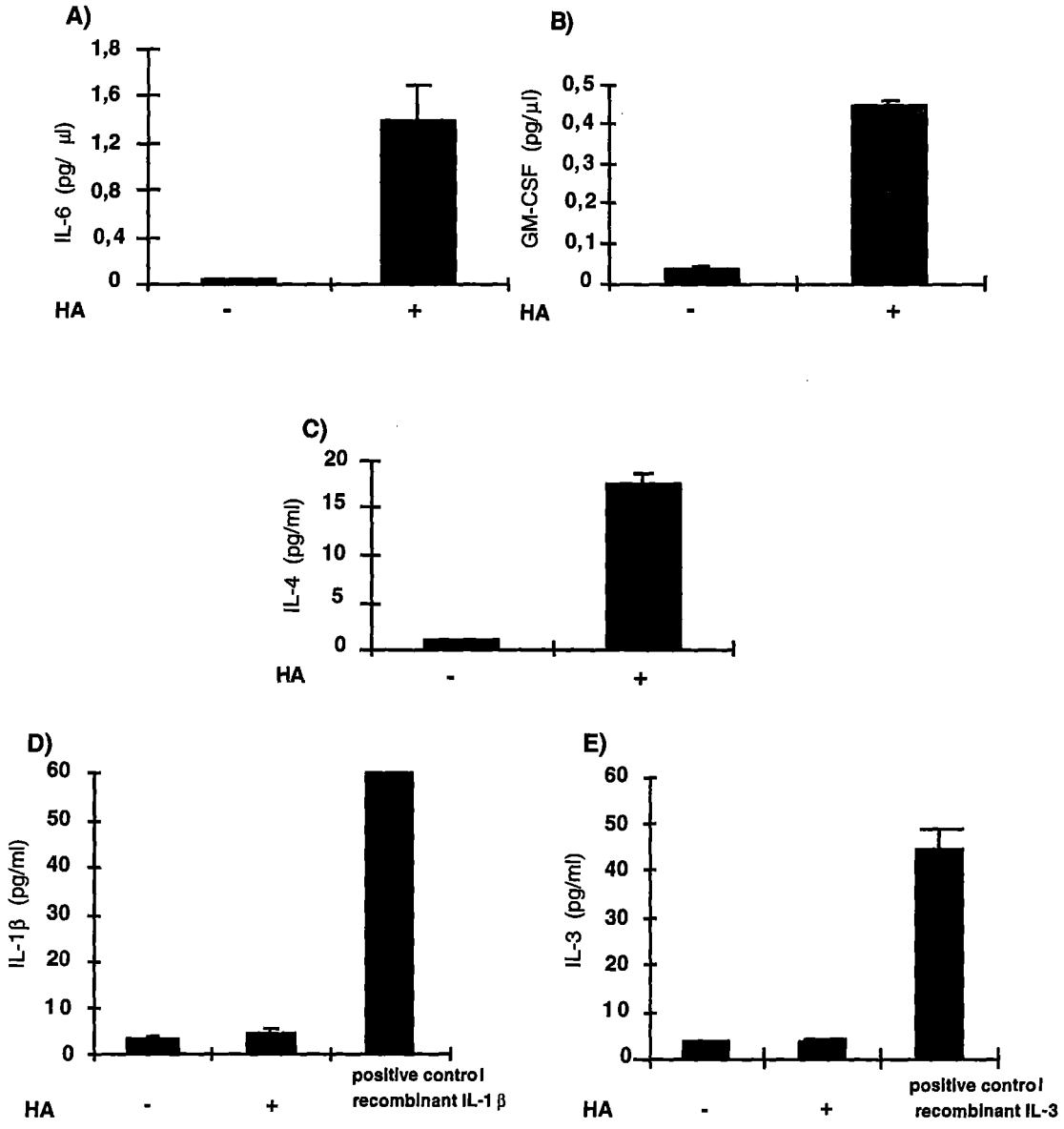


Figure 7. HA stimulates the production of IL-6, GM-CSF and IL-4 cytokines in MS-5 cells. *Cells were allowed to grow up to 70% confluence and were stimulated for 24 hours with 100 µg/ml of HA. The concentration of IL-6 (**A**), IL-4 (**B**), GM-CSF (**C**), IL-1β (**D**) and IL-3 (**E**) in the supernatants was determined by ELISA kits. SD was calculated from triplicates.*

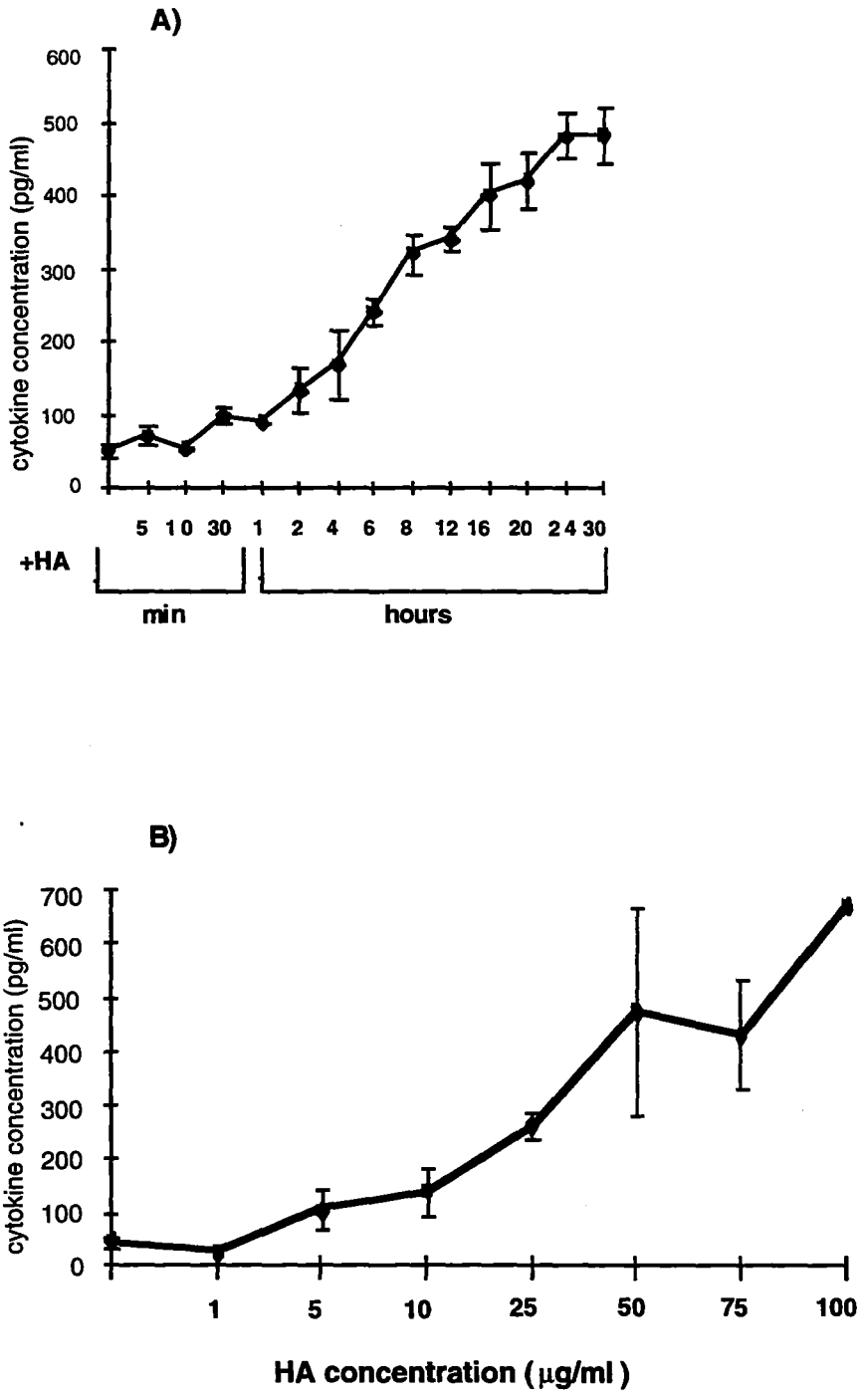


Figure 8. HA stimulates the production of cytokines in MS-5 cells in a time and dose dependent manner.

A) *Time dependent effect of HA on cytokine production.*

B) *Stimulation of MS-5 cultures with different HA dilutions.*

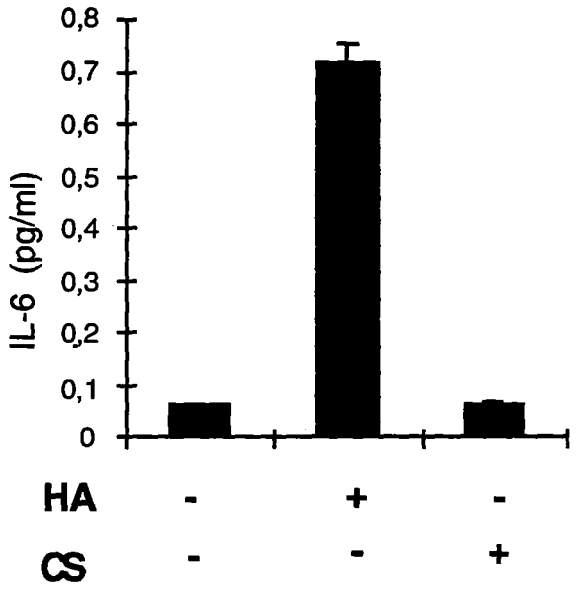


Figure 9. CS does not stimulate the production of cytokines in MS-5 cells. *Cells were allowed to grow up to 70% confluence and were stimulated for 24 hours with 100 µg/ml of HA and CS. Cytokine concentration in the supernatants was measured by an ELISA kit. SD was calculated from results obtained from triplicates.*

3.1.4 MS-5 cells can be stimulated by immobilised HA

HA can induce cytokine release either by binding to and activating its receptor on the cell surface or by being uptaken into the cells by a process called endocytosis. To distinguish between these two possibilities, we performed an experiment using for MS-5 activation HA fixed to the culture plates and compared the effect with that of soluble HA. Cytokine production in the culture supernatants was measured by ELISA (Fig.10). Immobilised HA induced cytokine production similarly to soluble HA. BSA- covered and PBS-treated plates had no influence. The observation that immobilised HA is still able to induce cytokines in MS-5 cells, suggests the involvement of a receptor-ligand interaction and signalling from the cell surface.

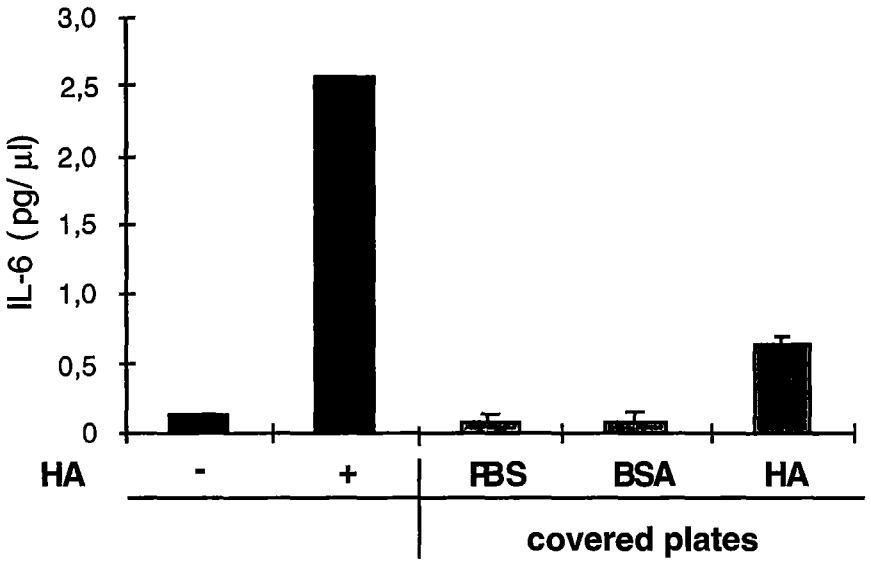
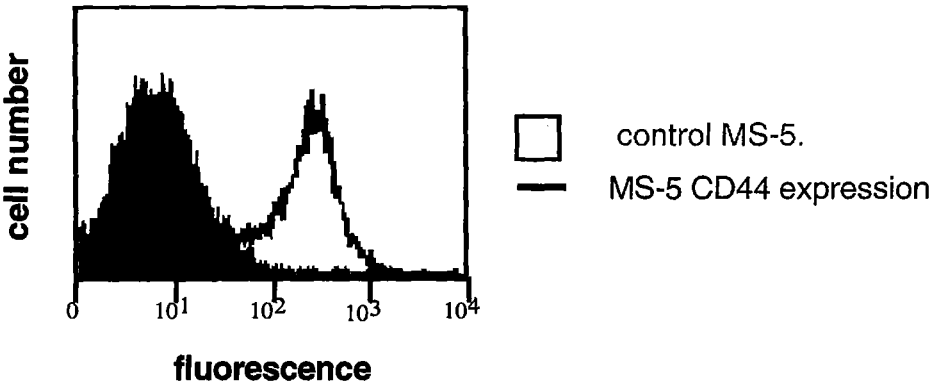


Figure 10. Immobilised HA can stimulate IL-6 production in MS-5 cells. *The plates were incubated O/N at 4°C with PBS, 1mg/ml BSA or 1mg/ml HA solution and washed 3 times with PBS. MS-5 cells were cultured on the HA- BSA- or PBS -covered plates for 24 hours. Cytokine concentration in the supernatants was measured by an ELISA kit. SD was calculated from results obtained from triplicates*

3.1.5 Is CD44 the receptor mediating cytokine induction?

Since CD44 is the most widely expressed HA receptor, we tested whether it accounts for the HA effect on MS-5 cells. We first looked at the expression of CD44 isoforms on MS-5 cells. As shown in Fig.11, a FACS analysis and a CD44 exon specific RT-PCR analysis revealed that MS-5 cells carry the CD44 standard isoform and the CD44v9 isoform.

A)



B)

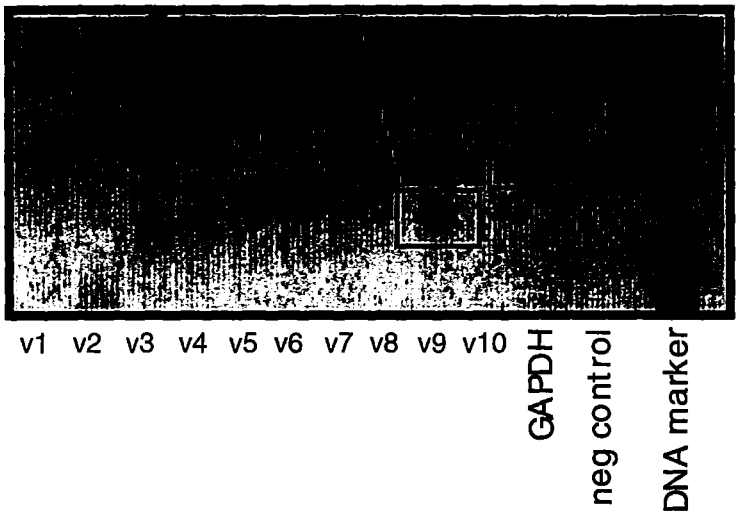


Figure 11. MS-5 cells express CD44 molecules on their surface.

A) *FACS staining with anti-CD44 IM7 antibodies.*

10^5 cells were stained with anti-CD44 antibodies. The fluorescence was analysed on a FACS-Star plus flow cytometer.

B) *CD44 exon-specific RT-PCR analysis.*

CD44 exon-specific RT-PCR analysis was performed as described in Materials and Methods. The PCR products were analysed by an agarose gel electrophoresis.

To answer the question whether MS-5 cells bind HA through CD44, we performed a FACS analysis with FITC labelled HA. The CD44 specificity of the binding was tested with antibodies that react with the HA-binding domain of CD44 (KM81) and thereby prevent HA binding. Pre-treatment of MS-5 cells with KM81 mAbs antibodies for 1 hour before addition of HA strongly decreased this HA-binding. Thus, we concluded that MS-5 cells bind HA mainly via CD44 (Fig.12).

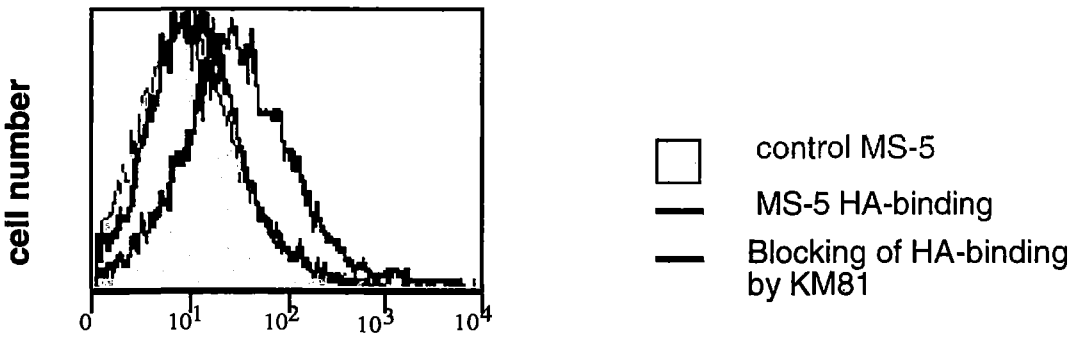


Figure 12. KM81 antibodies abrogate HA binding by MS-5 cells. *10^5 cells were stained with FITC-HA with or without pre-treatment with KM81 antibodies. The fluorescence was analysed on a FACS-Star plus flow cytometer*

To determine whether the binding of HA to CD44 is required for the induction of cytokines we investigated the effect of the anti-CD44 antibodies KM81 on HA-stimulated cytokine production. The MS-5 cells were pre-incubated with the antibodies for 30 min before HA treatment. The pre-incubation with antibodies was done at 37¡C or on ice (to avoid the possible uptake of the antibodies by cells). 24 hours after stimulation with HA the culture supernatants were checked for the presence of cytokines.

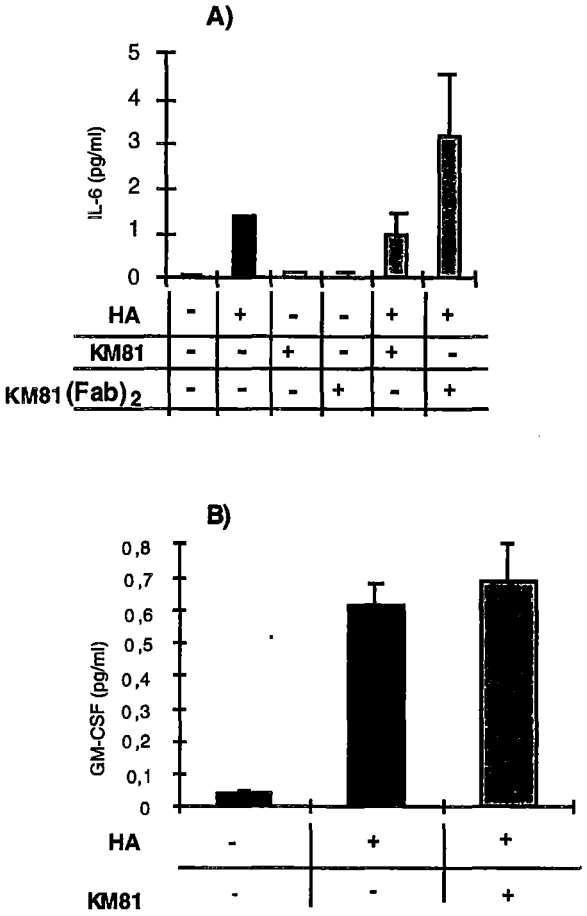


Figure 13. KM81 mAbs do not influence HA-mediated cytokine production. *MS-5 cells were grown up to 70% confluence, pre-treated for 1 hour with 20 µg/ml of KM81 and induced with 100 µg/ml of HA. After 24 hours the cytokine concentration in the supernatants was measured by an ELISA kit. SD was calculated from results obtained from triplicates.*

A) IL-6 B) GM-CSF.

We showed that under both conditions (37¡C or 4¡C), neither IL-6 induction (Fig.13) nor GM-CSF induction was repressed by the anti-CD44 antibodies. This observation suggests that, despite the involvement of CD44 in the HA binding, the HA- induced cytokine secretion in MS-5 cells seems to occur through a CD44 independent mechanism.

3.1.6 Possible involvement of another HA receptor in the induction of cytokines response

We investigated whether other proteins on the surface of MS-5 cells could bind HA. For this purpose we took advantage of the CPC (Cetylpyridinium chloride) precipitation method, which allows to pull down proteins that bind glycosaminoglycans (Sleeman *et al.*, 1993). Surface proteins on MS-5 cells

were biotinylated and protein lysates were used for the CPC precipitation with HA and with CS (as a control). Proteins bound to glycosaminoglycans were resolved on a SDS-PAGE gel under reducing conditions, blotted onto a membrane and visualised by means of HRP-conjugated antibodies (Fig.14).

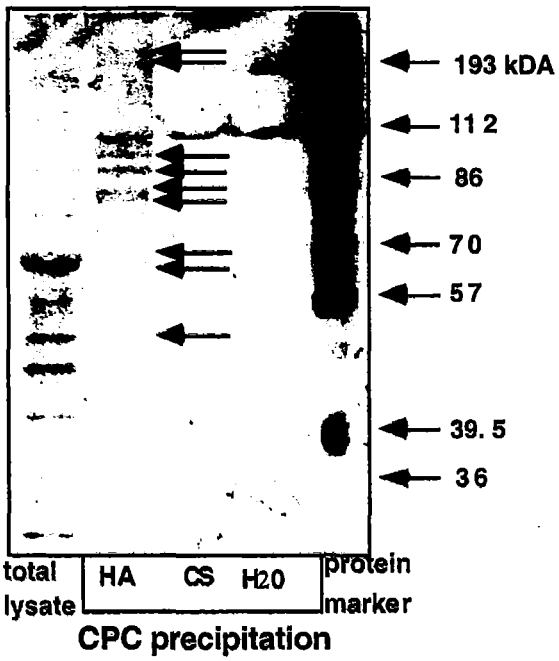


Figure 14. CPC precipitation of biotinylated surface proteins from the MS-5 cell line. *The cell surface proteins were biotinylated as described in Materials and Methods. Protein lysates were incubated with 1mg/ml of HA, CS or with H_2O. Bound proteins have been precipitated with CPC solution. Total cell lysate was used as a positive control.*

9 proteins from MS-5 cells seem to specifically bind HA as compared to CS and H_20 controls (Fig.14). They are putative receptors for HA and could mediate HA signalling leading to cytokine induction. To demonstrate the specificity of the CPC precipitation method, we examined whether CD44 is pulled down by HA among the other proteins. In this case the whole procedure was performed in non-reducing conditions and CD44 was visualised with the IM7 mAb. We observed that two CD44 isoforms can be CPC-precipitated from the MS-5 cells protein lysate among other HA-binding proteins (Fig.15).

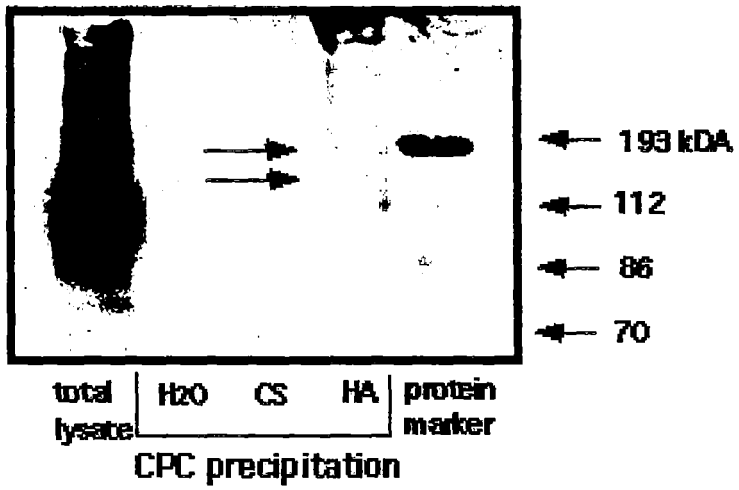


Figure 15. CD44 is CPC precipitated from the MS-5 cell line. *Protein lysates were incubated with 1mg/ml of HA, CS or with H_2O. Bound proteins have been precipitated with CPC solution. Total cell lysate was used as a positive control. The blot was probed with pan CD44 (IM7) antibodies.*

From those observations we conclude that not only CD44, but also other proteins on the MS-5 cell surface can bind HA and their binding might be decisive for the induction of cytokine production.

3.1.7 HA stimulation of MS-5 cells leads to rearrangment of cytoskeleton.

Extracellular signals affecting cells often result in cytoskeleton rearrangements. In order to investigate whether the cytoskeleton structure of MS-5 cells is changed upon HA treatment we stained the cells with phalloidine-rhodamine antibodies before and after stimulation with HA. Indeed the treatment of MS-5 cells with HA was accompanied by a rearrangement of the cytoskeleton resulting in the appearance of lammellopodias and membrane ruffles. The effect was observed already 15 min after HA-stimulation and lasted up to 16 hours (Fig.16).

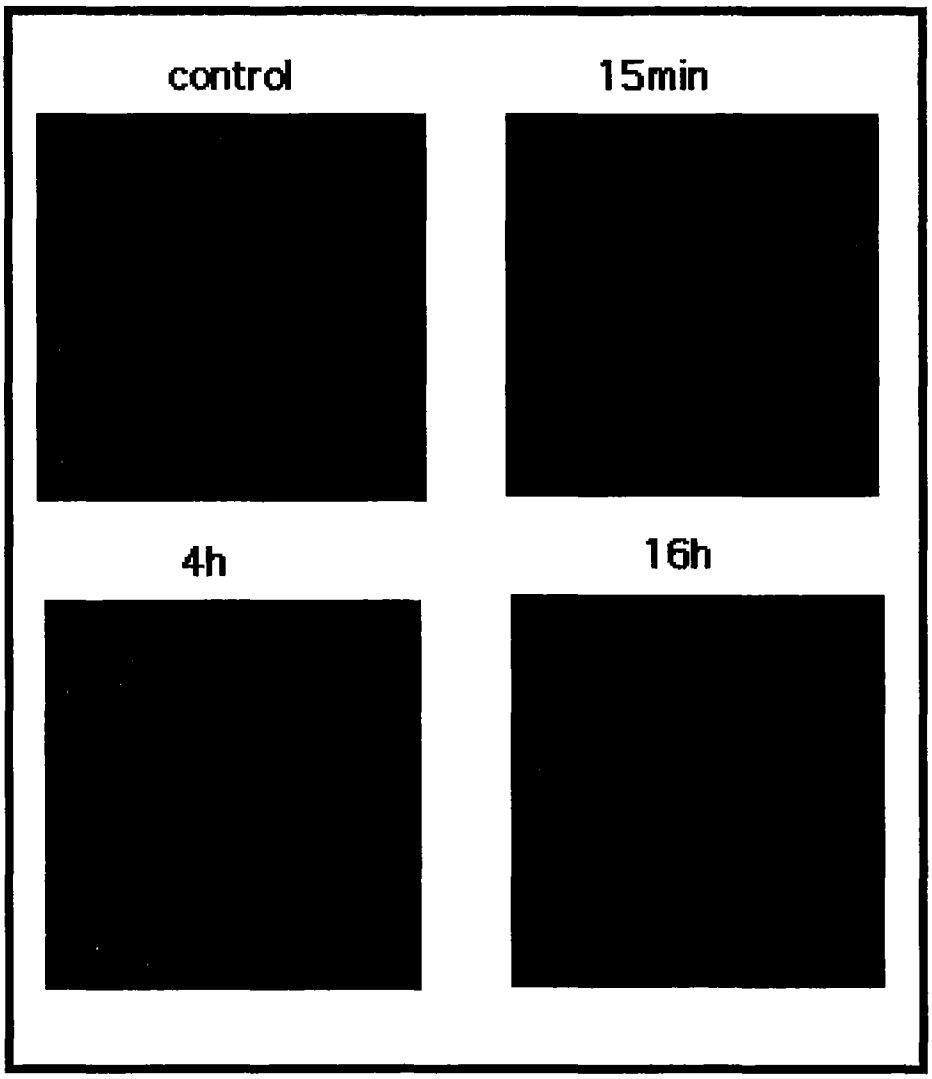


Figure 16. HA-mediated cytoskeleton rearrangement in MS-5 cells. *MS5 cells were incubated with 100 μg/ml of HA for different times (15 min, 4 hours, 16 hours). Actin staining was performed with phalloidine-rhodamine and was evaluated by fluorescence microscopy.*

3.1.8 Involvement of ERK and p38 in HA- signalling.

Extracellular signals received by receptors are further transmitted to the nucleus via intracellular signalling cascades finally leading to changes in gene expression. These cascades include activation of mitogen activated protein kinases (MAPKs) (Su *and* Karin, 1996) (Fig.17). MAPKs constitute a group of serine/treonin specific, proline directed protein kinases. So far several distinct MAPKs in vertebrates have been identified including ERK1/2, SAPK/JNK and p38.

We investigated the possible involvement of members of the protein kinase family in mediating cytokine expression in MS-5 cells upon HA treatment. The activation of kinases was monitored by their phosphorylation status. MS-5 cells were incubated in serum-free medium for 24 hours and induced with HA for different times. HA treated and non-treated cells were lysed and samples were investigated for MAPK activity using antibodies specific for the phosphorylated forms of ERK1/2, p38 and SAPK/JNK.

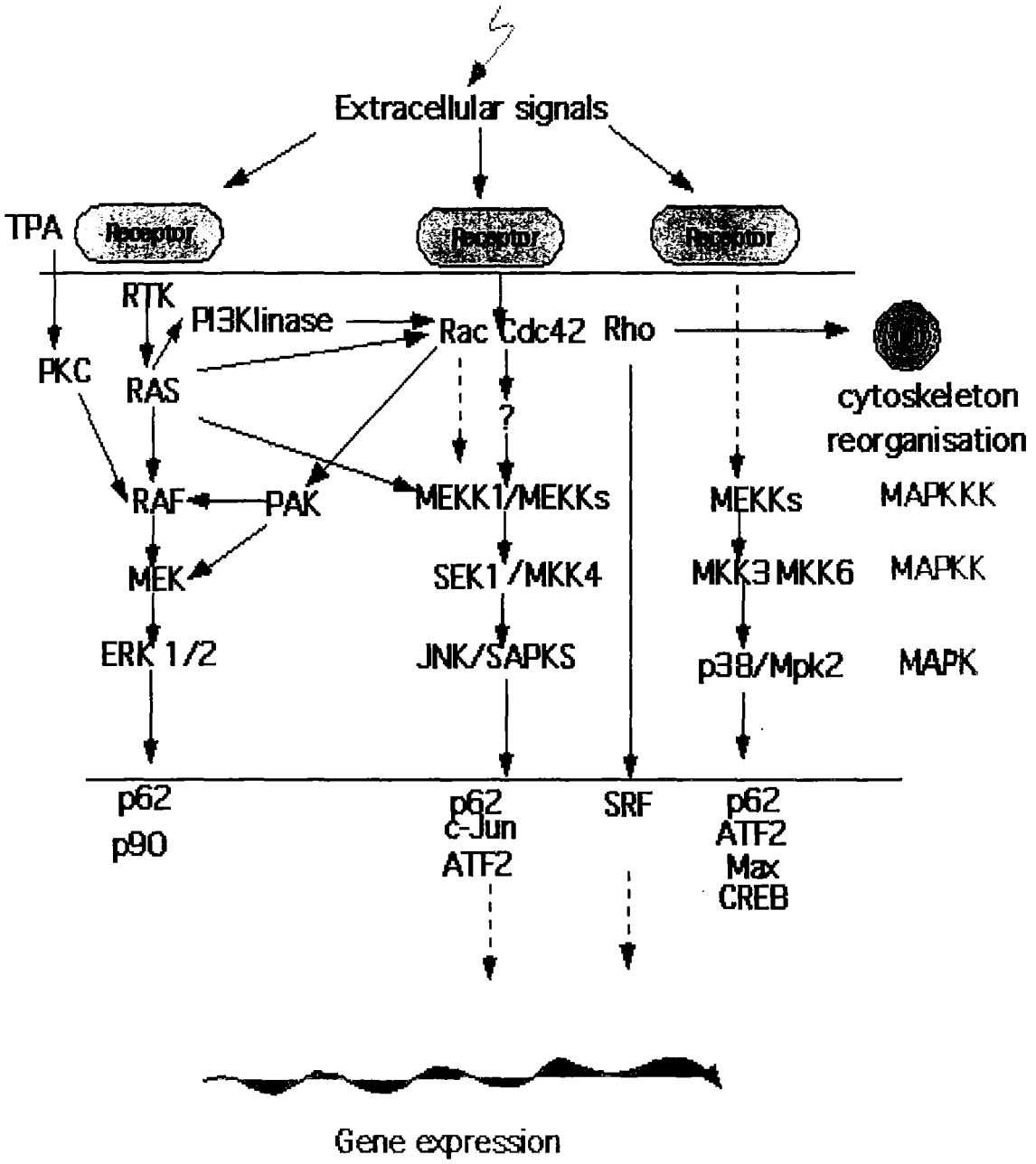


Figure 17. Schematic representation of eukaryotic MAPK signal transduction pathways (ERK1/2, JNKs/SAPKs and p38s pathway).

ATF: activating transcription factor, ***ERK:*** *extracellular signal-regulated kinase,* ***JNK:*** *Jun-N-terminal kinase,* ***MAPK:*** *mitogen activated protein kinase ,* ***MAPKK:*** *MAP-Kinase-Kinase,* ***MAPKKK:*** *MAP-kinase-kinase-kinase,* ***MEK:*** *MAPK/ERK-kinase,* ***MEKK:*** *MEK-kinase,* ***MKK:*** *MAP-kinase-kinase,* ***PAK:*** *p21-activated protein kinase,* ***RTK:*** *Receptor-Tyrosin-Kinase,* ***SAPK:*** *Stress-activated Protein Kinase,* ***SEK:*** *SAPK/erk-Kinase,* ***TPA:*** *12-0-tetradecanoyl-phorbol-13-Acetat.*

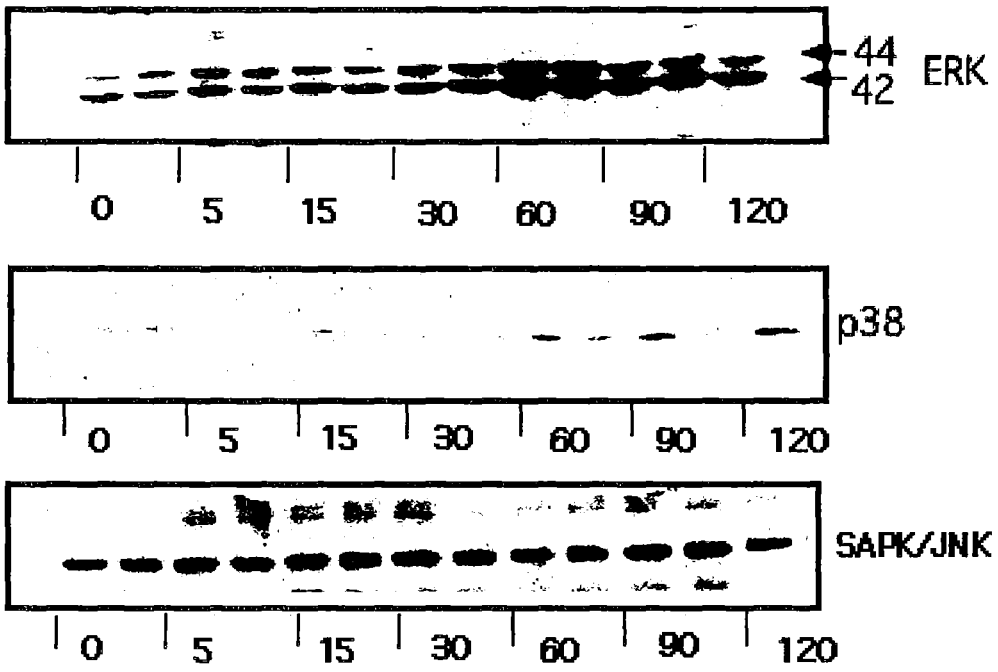


Figure 18. HA-induces activation of ERK and p38 MAPKs in MS-5 cells and does not influence SAPK/JUN kinase. *MS-5 cells were allowed to grow up to 70% confluence and were starved for 24 hours. Cells were incubated with 100 μg/ml of HA for different times and lysed with protein sample buffer. The samples were loaded on the SDS PAGE gel in duplicates and analysed by standard western blot procedure with anti- phosphospecific a)ERK, b)p38 or c)SAPK/JUN Kinase antibodies.*

SAPK/JNK was not influenced by the HA-treatment (Fig.18). In contrast, ERK1/2 and p38 became activated upon HA stimulation (Fig.18). The period of activation for the kinases was between 5-30 and 60-90 min for ERK1/2 and between 60-90 min for p38.

3.1.9 Kinases inhibitors prevent HA-mediated cytokine induction

HA treatment of MS-5 cells leads to the activation of ERK and p38 kinases, two kinases that belong to different signal transduction pathways (Fig.17). Using specific kinase inhibitors, we made an attempt to find out which pathway is directly involved in the upregulation of the cytokine production. The MS-5 cells were pre-incubated with different inhibitors for 30 min before HA induction. The culture supernatants were checked for the presence of cytokines. Wortmanin, a specific inhibitor of PI3 kinase (Okada *et al.*, 1994) did not influence IL-6 and GM-CSF cytokine production. The specific p38 inhibitor SB203580 (Simon *et al.*, 1998) down regulated GM-CSF production but did not influence the IL-6 response (Fig.19). ERK activation is mediated through specific protein kinases MAPK/ERK kinases (MEK) (Fig.17). The specific MEK1 inhibitor PD-98059 (Simon *et al.*, 1996) strongly decreased IL-6 production and only partially affected the production of GM-CSF (Fig.19). To further confirm the involvement of the ERK pathway in IL-6 production induced by HA, we also used the inhibitor UO126, that also inhibits the ERK activating MAPK kinase MEK (Favata *et al.*, 1998). UO126 treatment completely abrogated HA-mediated IL-6 induction (Fig.20).

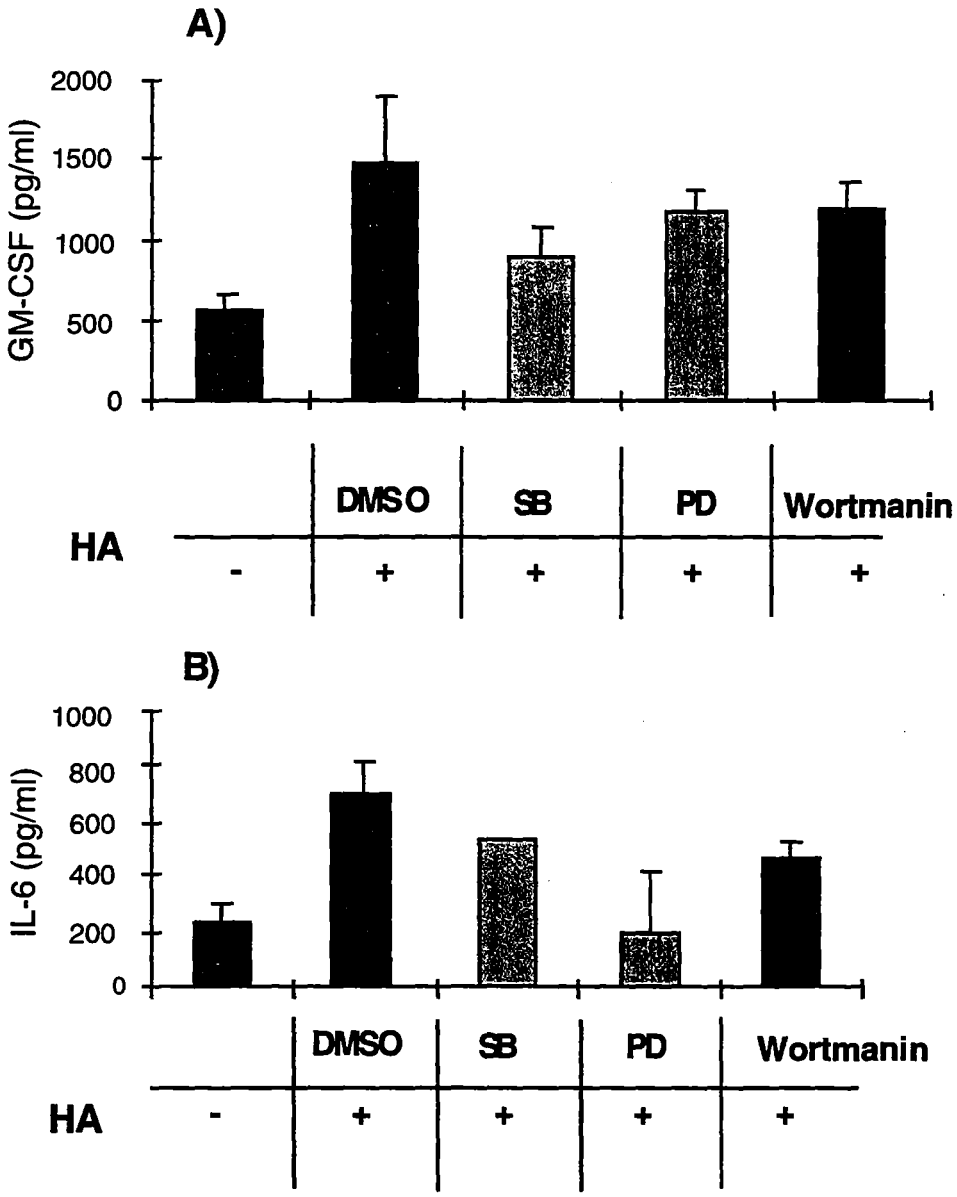


Figure 19. Influence of inhibitors of signalling cascade on HA-induced cytokine production by MS-5 cells *MS-5 cells were grown up to 70% confluence and starved for 24 hours in medium containing 0% FCS. Cells were pre-treated for 30 min with SB203580 (25 μM), PD98059 (50 μM) or Whortmanin (1 μM) and induced with 100 μg/ml HA. Cytokine concentration in the supernatants was measured by an ELISA kit.*

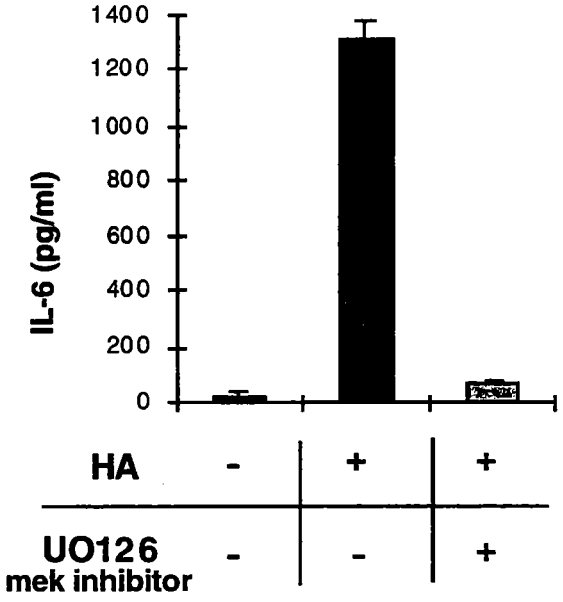


Figure 20. HA-induced IL-6 production is inhibited by the ERK pathway inhibitor UO126. *MS-5 cells were grown up to 70% confluence. Cells were pre-treated for 30 min with UO126 and induced with 100 µg/ml of HA. Cytokine concentration in the supernatants was measured by an ELISA kit.*

These results demonstrate the involvement of MAPKs in the HA-induced cytokine response. We conclude that the IL-6 stimulating HA signal in MS-5 cells is transferred mainly through the MEK-ERK pathway and the GM-CSF signalling possibly involves p38 activation.

3.1.10 Raf dominant-negative mutant downregulates IL-6 production

To further dissect the signalling pathway involved in the HA-mediated IL-6 activation, we examined the contribution of Raf, a kinase upstream of MEK. Its possible involvement was tested using a dominant-negative mutant (Fig.21).

MS-5 cells were transiently transfected with this Raf-1 -dominant-negative mutant or with a Raf-1-wild type construct, before stimulation with HA. 24 hours after HA addition the supernatants were tested for the amount of cytokine produced.

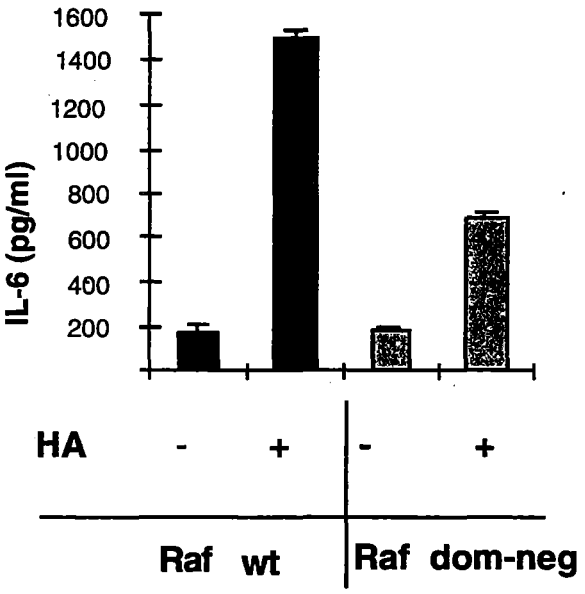


Figure 21. Dominant negative mutant of Raf-1 inhibits HA-mediated IL-6 secretion. *MS-5 cells were transiently transfected by electroporation with Rafwt and RafC4 dominant-negative construct. Cells were cultured 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours with 100 µg/ml of HA. Cytokine concentration in the supernatants was measured by an ELISA kit.*

The Raf dominant-negative mutant reduced the HA-mediated IL-6 response in MS-5 cells by approximately 50%. The transfection efficiency of MS-5 cells in those experiments was approximately 42% (as controlled by co-transfection of cells with the green fluorescence protein (GFP). Since only the transfected cells can show the Raf dominant-negative effect, we conclude that IL-6 induction in all transfectants is completely abrogated. Thus, our results place HA-signalling leading to IL-6 production in the Raf-MEK-ERK transduction pathway.

3.1.11 HA induces IL-6 mRNA expression in MS-5 cells at the level of gene transcription

To examine whether the increase of IL-6 and GM-CSF expression upon HA treatment is due to upregulation of transcription and not due to RNA stabilisation, we preincubated cells before HA-stimulation with the transcription inhibitor actinomycin D. Actinomycin D could completely block the ability of HA to increase the amount of IL-6 specific RNA. This result indicates that HA regulates IL-6 in MS-5 cells at the level of transcription. In contrast, pre-treatment of the MS-5 cells with actinomycin D did not significantly affect HA- dependent GM-CSF increase (Fig.22). Therefore GM-CSF production seems to be due to RNA stabilisation.

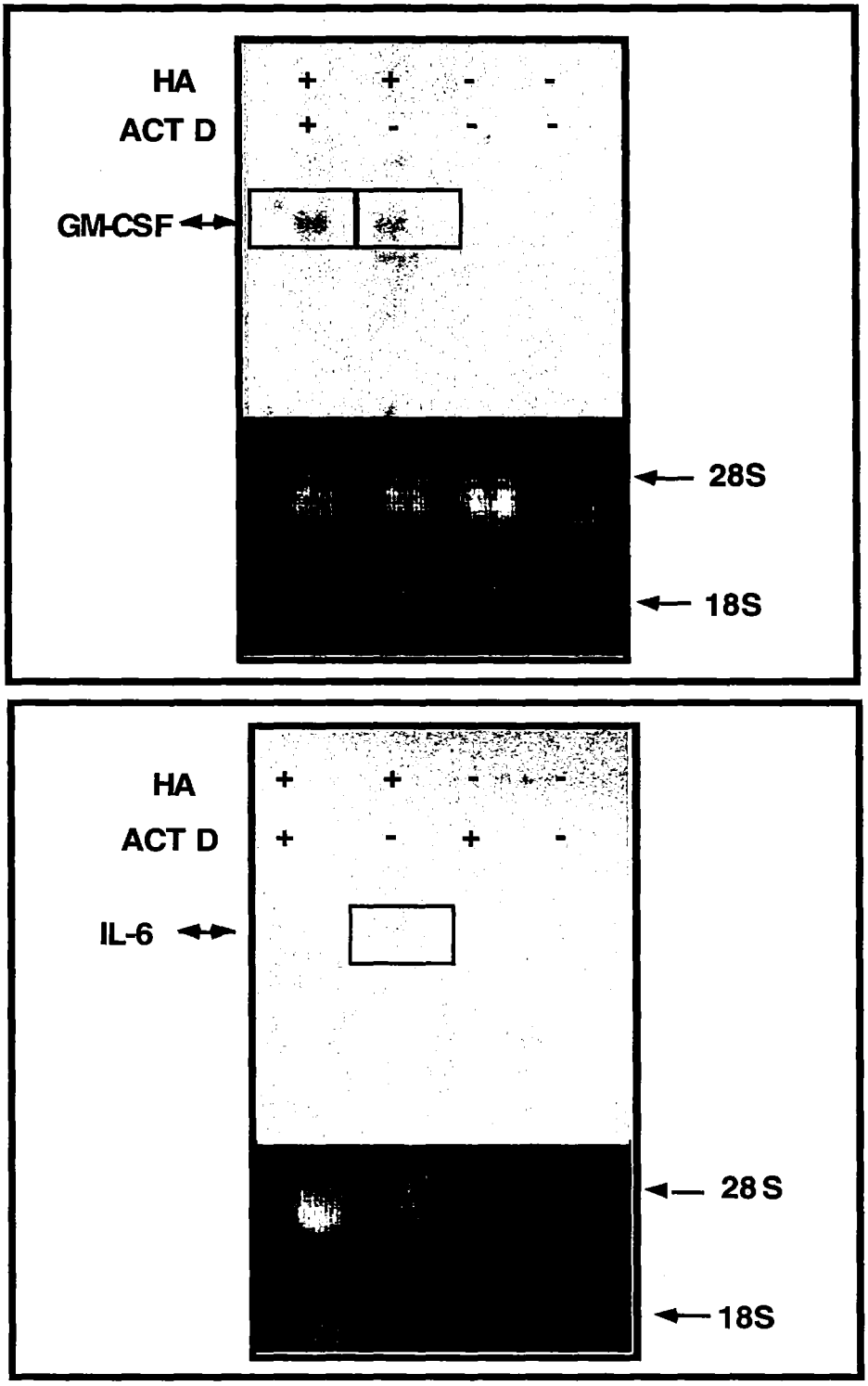


Figure 22. HA induces IL-6 expression on the transcriptional level. *MS-5 cells were grown up to 70% confluence and starved for 24 hours in medium with 0% FCS. Cells were pre-treated with actinomycin D and induced with 100 µg/ml of HA. After 6 hours RNA was isolated and Northern blot analysis was performed.*

A) *GM-CSF*
B) *IL-6*

3.1.12 HA enhances IL-6 promoter activity in MS-5 cells

To further confirm that induction of IL-6 by HA is the result of a transcriptional enhancement, we tested whether transfected IL-6 promoter constructs also respond to HA treatment. A full length promoter construct was cloned from MS-5 DNA using specific 5' and 3' primers (see Material and Methods) and fused to the luciferase gene sequence as a reporter gene. MS-5 cells were transiently transfected by electroporation with this IL-6 promoter construct (or vector control) and stimulated with HA for 24 hours. Cell lysates were examined for the amount of luciferase activity. We observed that HA could indeed activate the exogenous IL-6 promoter construct (pIL6luc(1.3)). Surprisingly, a smaller (602 bp) IL-6 promoter was stronger induced by HA than the longer promoter construct, which could indicate the existence of a negative regulatory cis- elements within the region —602bp to -1.3 kb. In further experiments we used the construct pIL6luc(-602) as an entire promoter construct because of its maximal inducibility (Fig.23). Parallel to the activation of IL-6 promoter constructs we always tested the activation of the endogenous IL-6 gene by an ELISA assay.

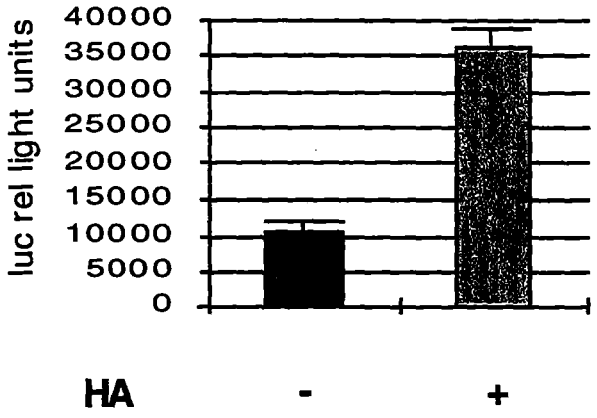


Figure 23. HA enhances IL-6 promoter activity in MS-5 cells. *MS-5 cells were transiently transfected by electroporation with IL-6 promoter luciferace reporter construct. Cells were cultured 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours with 100 µg/ml of HA. After stimulation cells were lysed and analysed for the amount of luciferase protein produced. The mean and SD represent three identical experiments.*

To define which of the sequences in the IL-6 promoter account for the HA response, we transiently transfected MS-5 cells with IL-6 promoter constructs containing further truncations at the 5'-end: pIL6luc(-602), pIL6luc(-298), pIL6luc(-235), pIL6luc(-169), pIL6luc(-122), pIL6luc(-60). Deletions up to —169bp in the IL-6 promoter did not significantly reduce HA-induced IL-6 promoter activity. Further deletions, to —122 bp and to —60 bp (pIL6luc(-122) and pIL6luc(-60)), showed no HA response (Fig. 24).

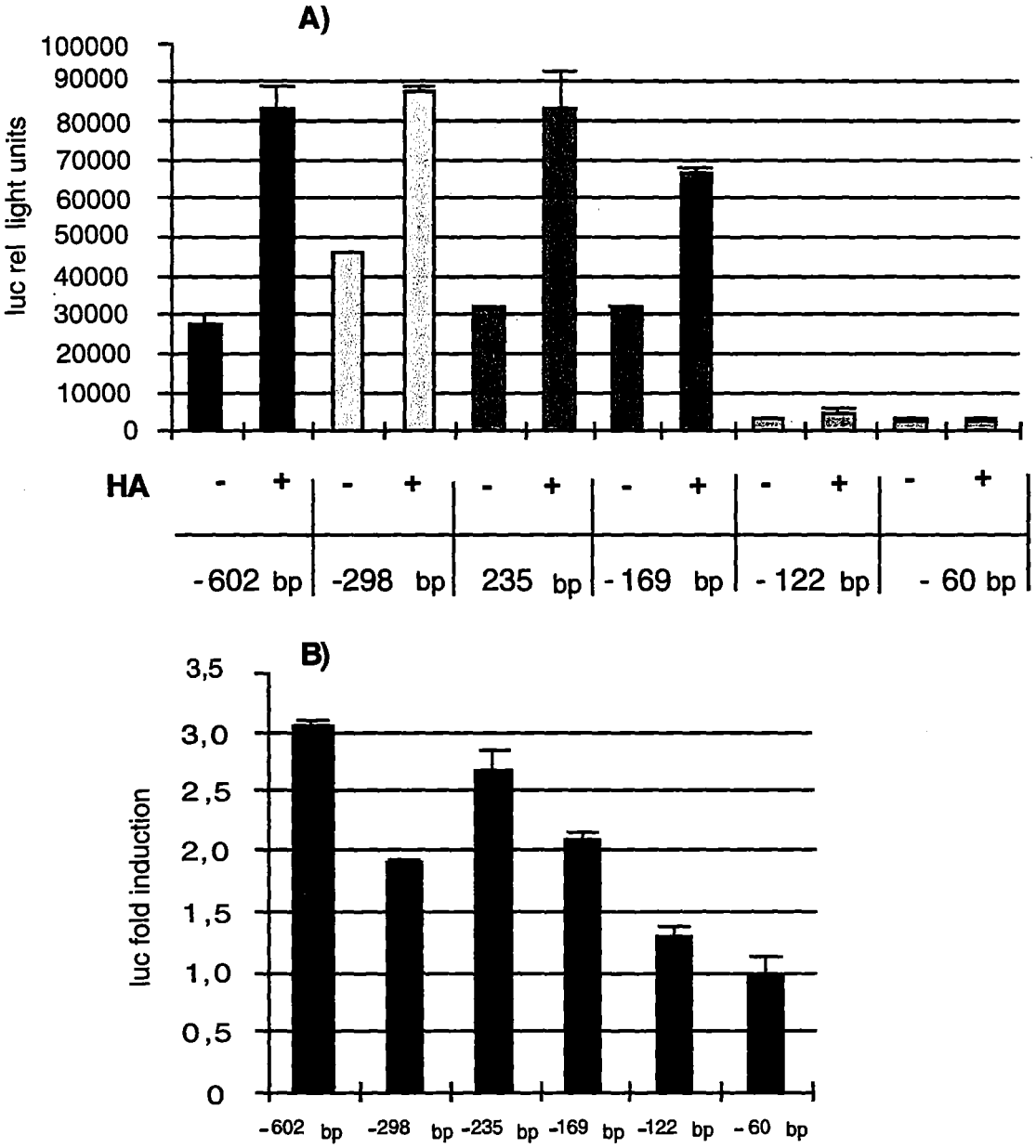


Figure 24. The deletion mutants of IL-6 promoter can abrogate HA-mediated IL-6 activation. *MS-5 cells were transiently transfected by electroporation with IL-6 promoter luciferace reporter deletion constructs. Cells were cultured 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours with 100 µg/ml of HA. After stimulation cells were lysed and analysed for the amount of luciferase protein produced. The mean and SD represent three identical experiments.*

A) *Represents the mean of luciferase relative units.*

B) *Represents the mean of fold induction.*

3.1.13 HA enhances the IL-6 promoter activity in MS-5 cells through NF-IL-6 and NF-κB regulatory cis-elements

In the region between —122bp and —60bp of the IL-6 promoter two potential binding sites for transcription factors are removed: at —122bp a NF-IL-6 binding site and at -60 bp a NF-κB binding site.
We therefore addressed the question whether both, the NF-IL-6 and the NF-κB site are important and necessary for HA-mediated IL-6 expression. We introduced into the 602 bp promoter construct point mutations in the NF-κB binding site and in the NF-IL-6 binding site which should inactivate these sites. The mutant constructs were transiently transfected into MS-5 cells and the expression of luciferase was tested upon HA treatment. Both mutants strongly abolished HA-mediated IL-6 promoter activation (Fig.25). This result demonstrates that the NF-IL-6 and the NF-κB regulatory cis-element are targets for HA-dependent activation. To test whether NF-κB suffices for activation we additionally transfected the MS-5 cells with a construct containing 3 times a repeat of an NF-κB cis-element (3xNF-kB construct).

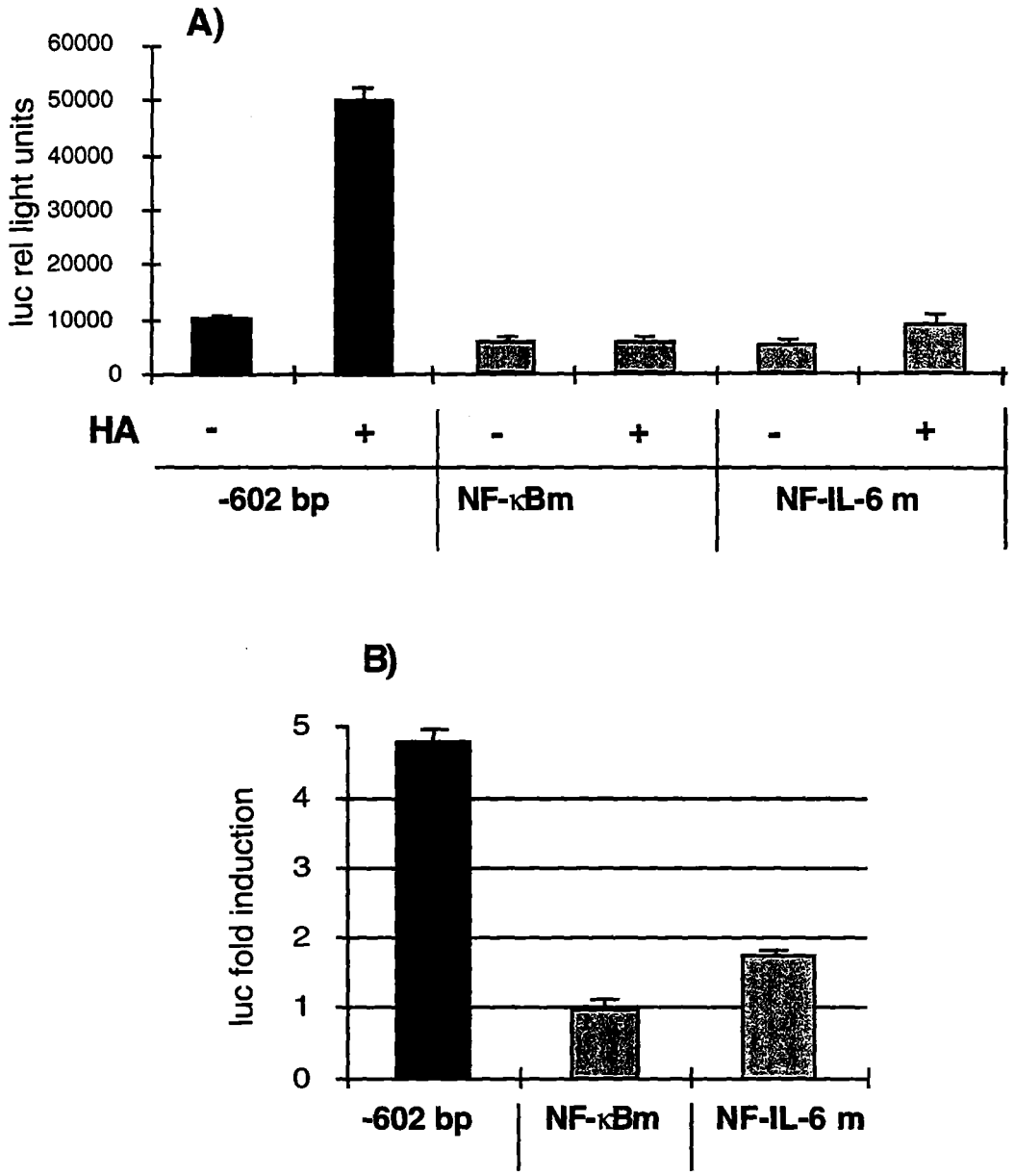


Figure 25. NF-κB and NF-IL-6 mutated cis- elements abrogate HA-mediated activation of IL-6 promoter constructs. *MS-5 cells were transiently transfected by electroporation with the IL-6 promoter luciferase reporter constructs pIL6luc(-602), pIL6lucNF-κBm, pIL6lucNF-IL-6m. Cells were cultured 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours with 100 μg/ml of HA. After stimulation cells were lysed and analysed for the amount of luciferase protein produced. The mean and SD represent three identical experiments.*

A) *Represents the luciferase relative units.*
B) *Represents the mean of fold induction.*

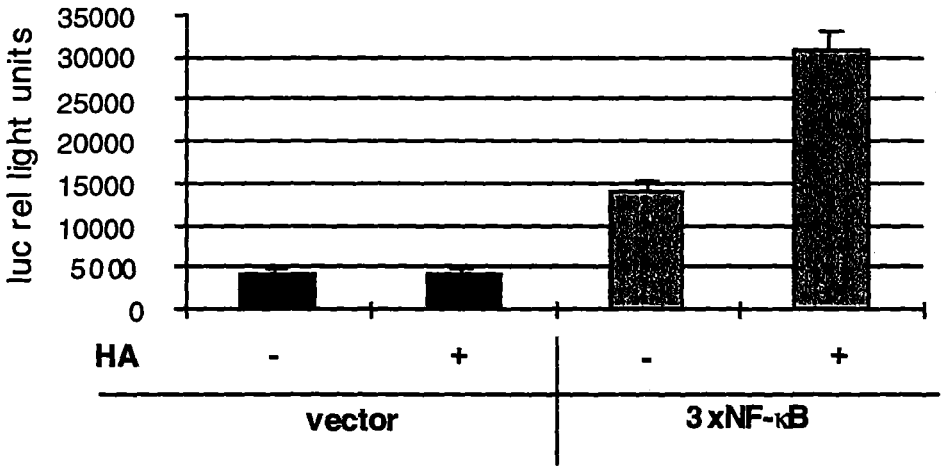


Figure 26. Construct containing a 3xNF-κB cis-element can be activated in MS-5 cells by HA-stimulation. *MS-5 cells were transiently transfected by electroporation with 3xNF-κB luciferace reporter construct. Cells were cultured 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours with 100 μg/ml of HA. After stimulation cells were lysed and analysed for the amount of luciferase protein produced. The mean and SD represent three identical experiments.*

The result of this experiment, illustrated in Fig.26, suggests that HA can directly increase NF-κB mediated transactivation. The NF-κB construct can be stimulated by HA, but not to such a high extent as the complete IL-6 promoter. However, this construct consists of 3 times repeated NF-κB cis-element. Thereby, in the endogenous IL-6 promoter the cooperative involvement of NF-IL-6 and NF-κB elements might be necessary.

Altogether our results indicate that:

A) HA is a potent inducer of the IL-6 gene promoter.

B) The synergistic transactivation by NF-IL6 and NF-κB factors might be a necessary and crucial event in HA-mediated IL-6 gene transcription.

3.2 Part 2: Signalling induced by interaction between MS-5 cells and TF-1 cells

Components of the ECM can be involved in cell- cell communication between hematopoietic progenitors and stromal cells. Previously the HA mediated adhesion of myeloid progenitor cell lines to the bone marrow stromal cell line MS-5 was demonstrated to be mediated by HA (Moll *et al.*, 1998).

Our observation about the regulatory role of HA on the stromal cells and the data showing that HA might be involved in progenitor -stromal cells adhesion motivated us to investigate a direct interaction between MS-5 stromal cells and $CD34^+$ TF-1 progenitor cells and later on possible involvement of HA in this process.

3.2.1 TF-1 cells stimulate MS-5 cells to produce cytokines

We investigated the cytokine production from the MS-5 cells before and after co-culturing with TF-1 cells. Since the MS-5 cells are murine cells and the TF-1 cells are of human origin, we used species-specific ELISA kits which allowed to distinguish which cell line is responsible for the cytokine production. Interestingly, we observed that upon co-culture with TF-1 cells, the MS-5 cell line can be activated to increase the amount of particular cytokines IL-6, GM-CSF and IL-4 (Fig.27).

The profile of cytokines upregulated upon TF-1 cells co-culturing with MS-5 cells- IL-6, IL-4 and GM-CSF- was similar to that observed upon HA-mediated activation of MS-5 cells. We also could not detect IL-3 or IL-1β production from MS-5 cells upon TF-1 co-culturing.

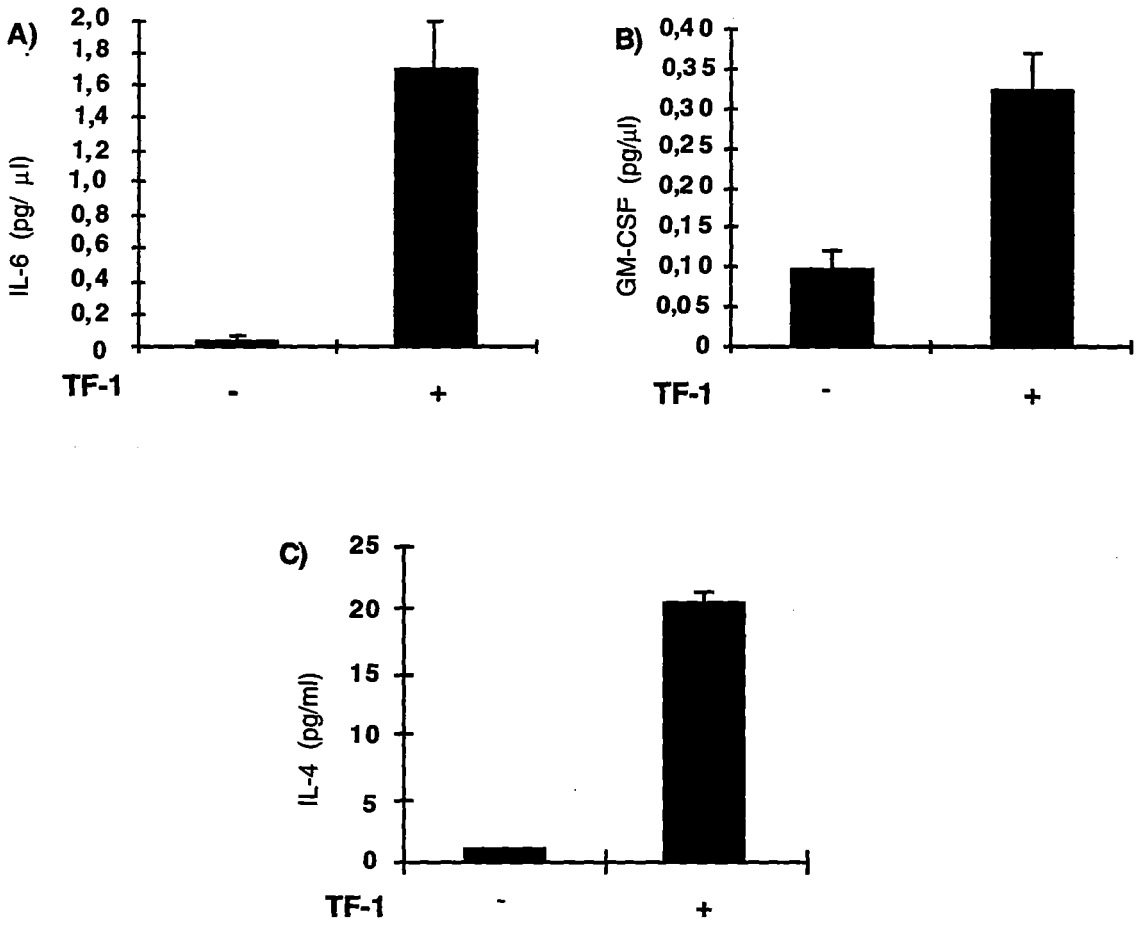


Figure 27. TF-1 cell line stimulates the production of IL-6, GM-CSF and IL-4 cytokines in MS-5 cells. *MS-5 cells were allowed to grow up to 70% confluence and were stimulated for 24 hours with 10^5 TF -1 cells. The concentration of IL-6 (**A**), IL-4 (**B**) and GM-CSF (**C**) in supernatants was determined by an ELISA kits. SD was calculated from results obtained from triplicates.*

3.2.2 Is a direct contact between MS-5 cells and TF-1 cells required for cytokine production?

MS-5 cells might be stimulated to produce cytokines by a direct cell contact with TF-1 cells or TF-1 cells can produce some soluble factors which are responsible for the TF-1- mediated cytokine response in MS-5 cells. To decide between these two possibilities, we performed a transwell assay. In this assay the two cell lines are separated by a filter. MS-5 cells were

cultured in 6-well culture plates up to 70 % confluence. Then the TF-1 cells were added to the plates either directly (for control) or seeded on transwell filters (45µm). The cytokine production was measured 24 hours later.

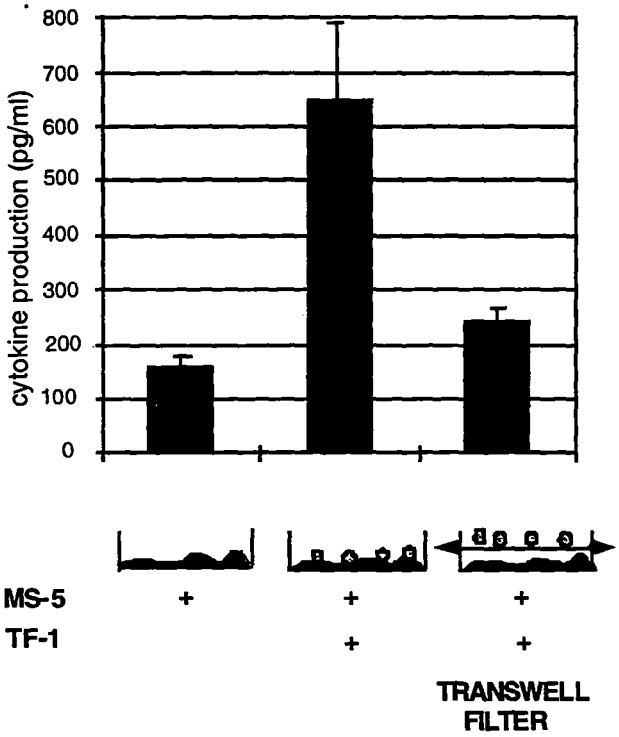


Figure 28. Transwell assay. *MS-5 cells were grown up to 70% confluence and pulsed by co-culture with TF-1 cells in the presence or the absence of a transwell filter. The concentration of cytokine in the supernatants was monitored by ELISA after 24 hours. Pure TF-1 culture was used as a negative control. SD was calculated from results obtained from triplicates.(GM-CSF kit).*

As already described the direct contact between TF-1 and MS-5 cells results in cytokine induction. The abrogation of the direct interaction between TF-1 and MS-5 cells abolished the TF-1- dependent cytokine induction in MS-5 cells (Fig.28).

3.2.3 Effect of suramin on TF-1 stimulated cytokine production

To test whether surface receptor contacts are required for the cytokine induction we tested the effect of suramin, an inhibitor of receptor activation (Stein, 1993), on TF-1-induced cytokine release. Pre-treatment of MS-5 cells

with 3 mM of suramin prior to TF-1 addition completely abolished the TF-1 effect (Fig.29).

Taken together, our results suggest that the induction of cytokines in the stromal MS-5 cells upon TF-1 addition is dependent on a direct cell-cell contact via surface receptor(s).

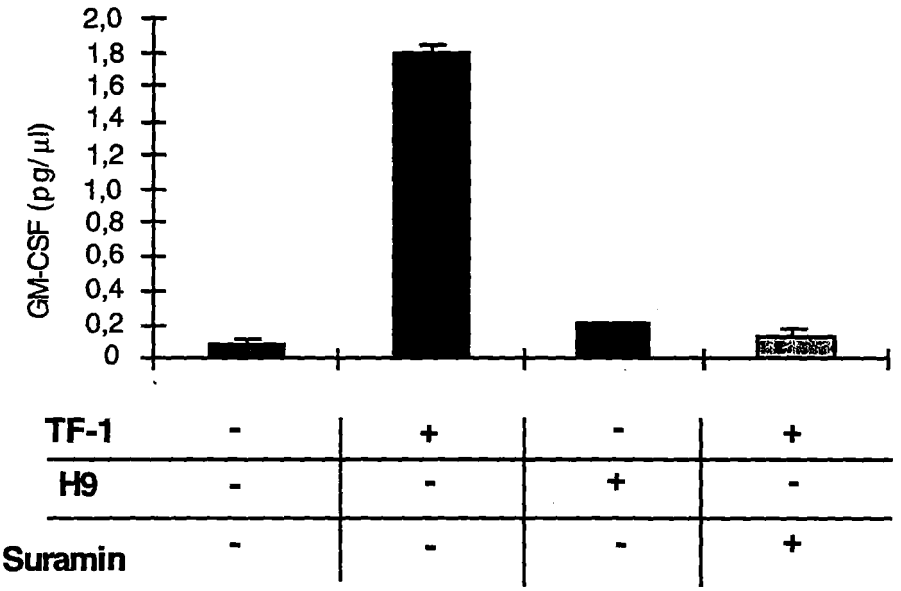


Figure 29. TF-1 mediated activation of MS-5 cells can be blocked by suramin. *MS-5 cells were grown up to 70% confluence. 10^5 TF-1 or H9 cells (as a control) were co-cultured with a MS-5 feeder layer with or without suramin pre-treatment. Cytokine concentration in the supernatants was measured by an ELISA kit. SD was calculated from results obtained from triplicates.*

3.2.4 CD44 antibodies have no influence on TF-1 triggered cytokine response in MS-5 cells

CD44 was previously shown to be implicated in the processes of cell adhesion (for review see Simmons *et al.*, 1997). Moreover the HA affinity for some progenitor cell lines was exclusively mediated by CD44 (Moll *et al.*, 1998). Therefore we investigated the expression of CD44 standard and variant forms on the surface of TF-1 cells by FACS analysis. $5x10^5$ cells were incubated for 30 min on ice with different anti-CD44 antibodies. The

result of this experiment is summarised in a Tab.1. The TF-1 cells carry the standard and several variant epitopes of CD44 molecule.

CD44 epitopes expressed by TF-1 cells	**Anti-CD44 antibodies used for the FACS staining.**
CD44st	*SFFpan*
CD44v5	*VFF8*
CD44v7	*VFF9*
CD44v9	*VFF14*
CD44v7-8	*VFF17*
CD44v6	*VFF18*

Tab 1. The expression of CD44 epitopes on TF-1 cells. *10^5 cells were stained with different anti-CD44 antibodies. The antibodies demonstrating positive staining are summarised in the table according to the CD44 epitopes they recognise.*

Next we tested whether a pre-treatment of cells with anti-CD44 antibodies had an effect on cytokine induction. Cells were pre-treated with anti-CD44 antibodies for 30 min before co-culture. The cytokine production in the supernatants was measured 24 hours later. Using anti-CD44 pan antibodies and antibodies against all expressed variant epitopes, we demonstrated that CD44 molecules do not play a role in TF-1 induced cytokine release in MS-5 cells, similarly to the case of HA-mediated activation (Fig.30).

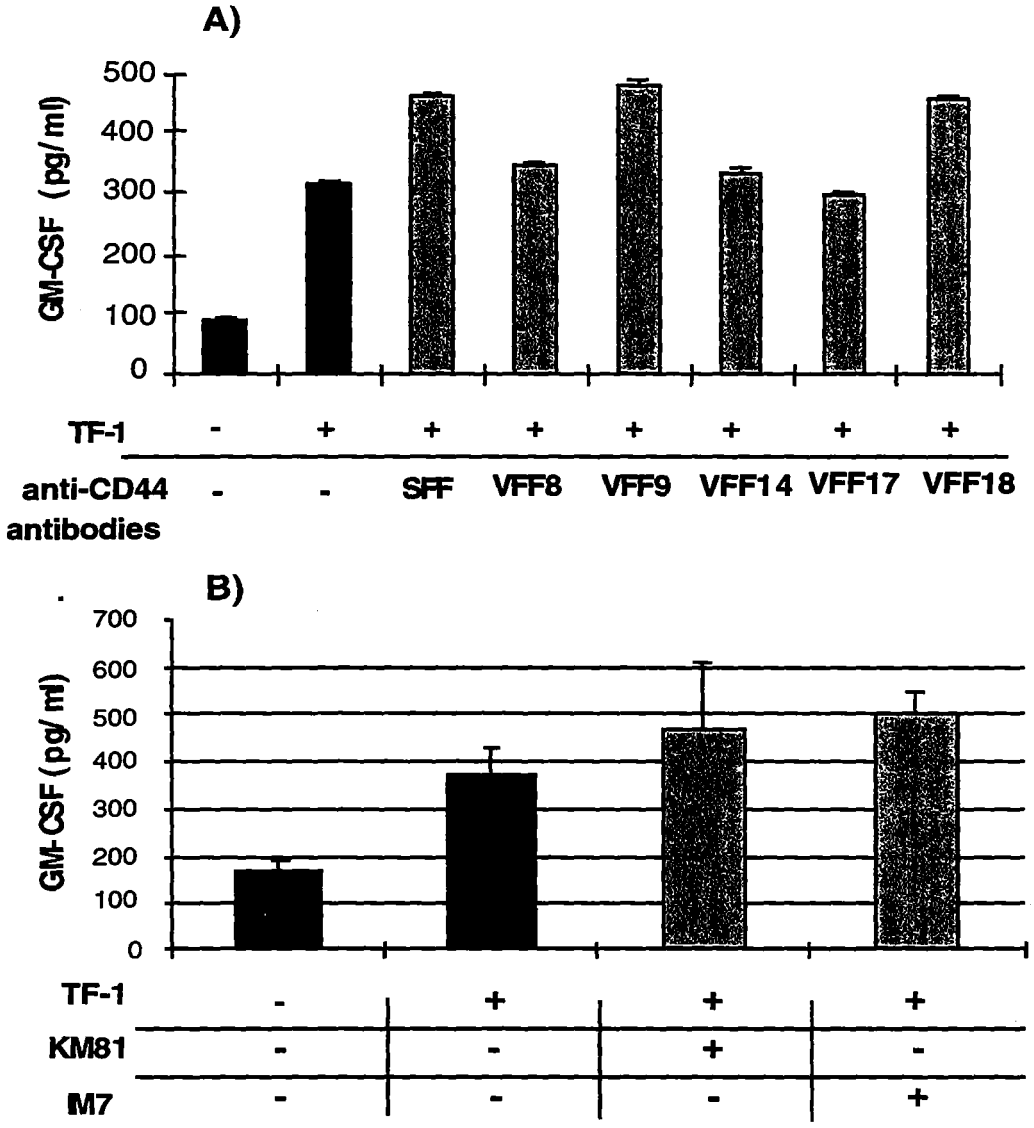


Figure 30. CD44 is not involved in TF-1-mediated cytokine production. *MS-5 cells were grown up to 70% confluence. MS-5 or/and TF-1 cells were pre-treated for 1 hour with 20 µg/ml of anti-CD44 antibodies and co-cultured for 24 hours. Afterwards, the cytokine concentration in the supernatants was measured by an ELISA kit.*

A) *human anti-CD44 antibodies SFF-CD44st, VFF8-CD44v5, VFF9-CD44v7, VFF14-CD44v10, VFF17-CD44v7-8, VFF18-CD44v6.*
B) *mouse pan anti-CD44 antibodies IM7 (cross-react with human CD44) and KM81.*
SD was calculated from results obtained from triplicates

3.2.5 Support of hematopoiesis by MS-5 cells requires cytokine production.

In order to compare the property of MS-5 cells to be activated to produce cytokines with their ability to support the survival of TF-1 cells, we established several MS-5 sub-lines by sub-cloning and checked these clones for the cytokine induction by TF-1 cells and HA. Among the sub-clones there were some that responded to TF-1 co-culturing but also some that did not respond (Fig.31). When tested for HA responsiveness, the same clones that were not induced for cytokine production by TF-1 also did not respond to HA. Moreover the inducibility of the clones positively correlated with their ability to support the growth of TF-1 cells (Fig.31).
Taken together these data indicate that the ability of the clones to be induced by the TF-1 cell line to produce cytokine correlates with their HA- mediated inducibility as well as with their property to support the proliferation of TF-1 cells, suggesting that in both cases a similar receptor or a similar signal transduction pathway might be involved.

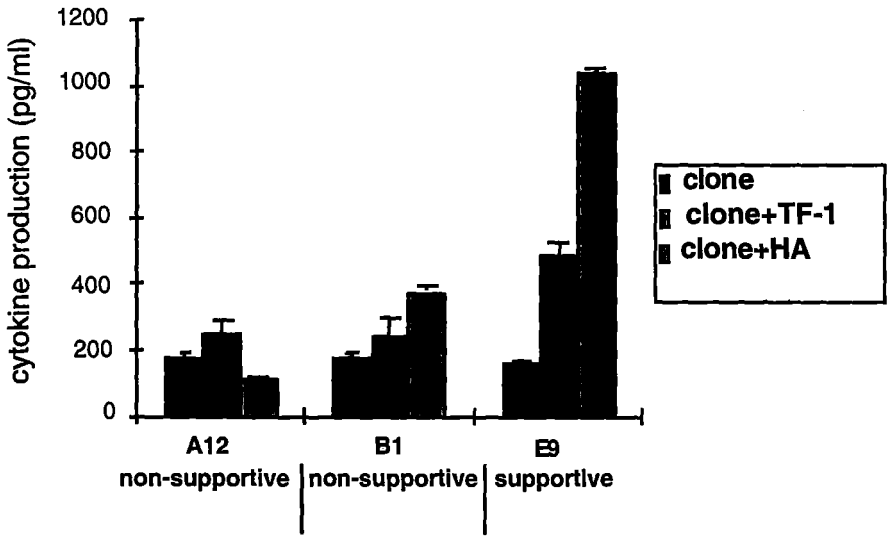


Figure 31. Responsiveness of MS-5 sub-clones to HA and TF-1 stimulation. *Different sub-clones of MS-5 cell line were grown up to 70% confluence and induced either with TF-1 cells or with HA. Cytokine concentration in the supernatants was measured by an ELISA kit. (GM-CSF kit).*

3.2.6 TF-1 cells activate the IL-6 promoter on the transcriptional level through NF-κB and NF-IL-6 cis-elements.

The similarities between TF-1-mediated and HA-mediated cytokine activation, e.g. the cytokine pattern obtained and the independence of CD44 suggest that TF-1 activation might occur by a similar mechanism than that involved in HA-mediated cytokine production. We therefore tested whether similar sequences within the IL-6 promoter are responsible for the TF-1 induced cytokine production by MS-5 cells. We transiently transfected the MS-5 cells with IL-6 promoter constructs containing truncations at the 5'.

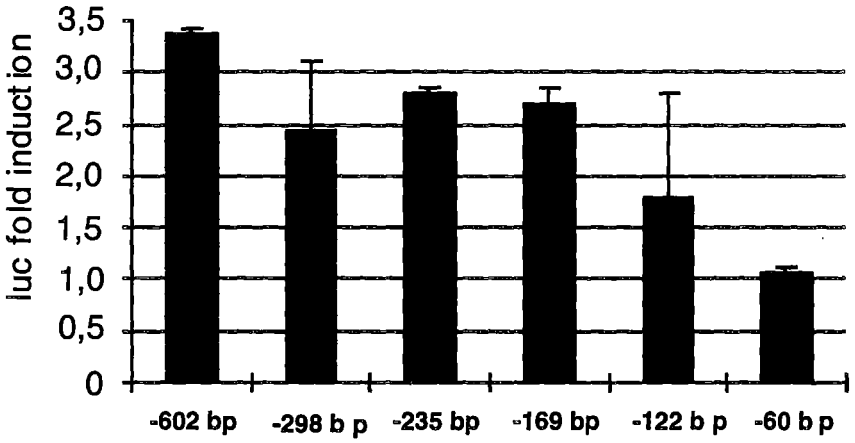


Figure 32. The deletion mutants of the IL-6 promoter can abrogate TF-1-mediated IL-6 activation. *MS-5 cells were transiently transfected by electroporation with IL-6 promoter luciferace reporter deletion constructs. Cells were cultured for 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours with 10^5 TF-1 cells. After stimulation cells were lysed and analysed for the amount of luciferase protein produced. The mean and SD represent three identical experiments. The figure represents the mean of fold induction.*

Thereafter, MS-5 cells were plated on 5 cm^2 dishes, starved and then treated with TF-1 cells. TF-1 cells were able to induce the IL-6 promoter activity (Fig.32).

The region between —169bpto -602bp did not contribute to the TF-1 mediated IL-6 activation, because deletion mutants covering this region did not show any change in inducibility by TF-1 cells as compared to the entire promoter (Fig.32). In contrast, the deletion mutants loosing NF-IL-6 and

NF-κB binding sites were not inducible any more. Similarly to HA, it seems that the MS-5 response to the TF-1 cells regulating the production of IL-6 depends on the activation of NF-IL-6 and NF-κB transcription factors. This was further confirmed by testing IL-6 promoter constructs containing point mutations in the NF-κB and NF-IL-6 sites. Both abrogated the response of MS-5 cells to TF-1 cells (Fig.33). Moreover TF-1 cells can activate a construct containing 3 times a repeat of an NF-κB cis-element (3xNF-kB construct), transiently transfected into MS-5 cells (Fig.34).

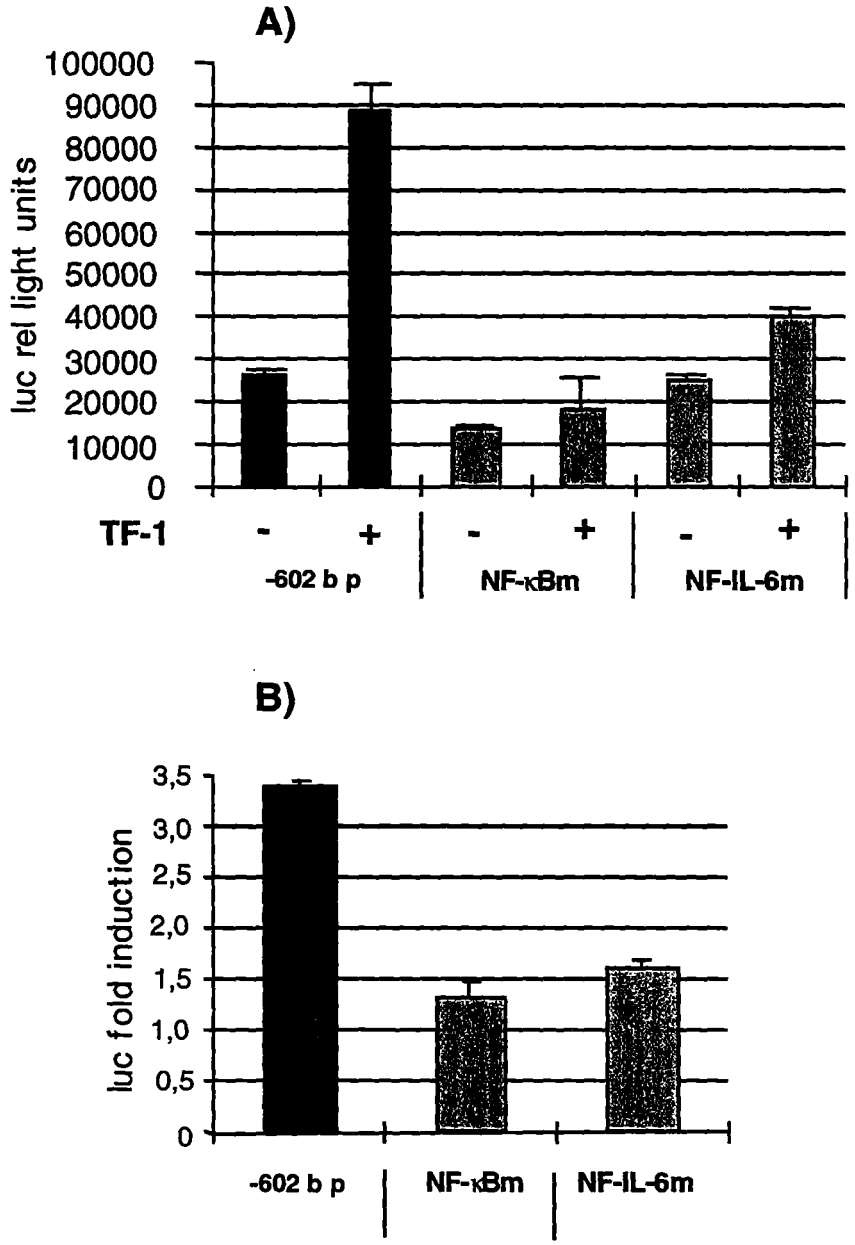


Figure 33. NF-κB and NF-IL-6 mutated cis- elements abrogate TF-1 mediated activation of IL-6 promoter constructs. *MS-5 cells were transiently transfected by electroporation with the IL-6 promoter luciferace reporter constructs pIL6luc(-602), pIL6lucNF-κBm, pIL6lucNF-IL-6m. Cells were cultured for 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours by co-culture with 10^5 TF-1 cells. After stimulation cells were lysed and analysed for the amount of luciferase protein produced. The mean and SD represent three identical experiments.*

A) *Represents the mean of luciferase relative units.*
B) *Represents the mean of fold induction.*

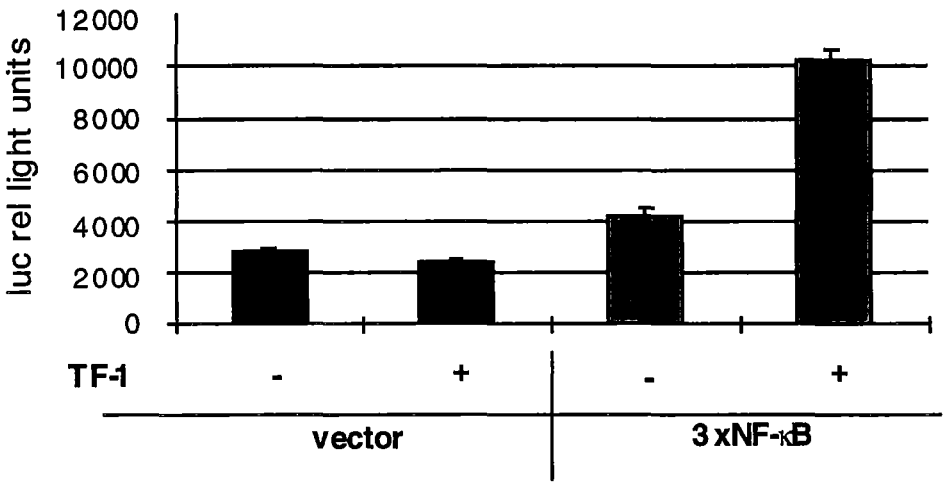


Figure 34. Construct containing 3xNF-κB cis-element can be activated in MS-5 cells by TF-1 stimulation. *MS-5 cells were transiently transfected by electroporation with 3xNF-κB luciferace reporter constructs. Cells were cultured 12 hours after transfection, starved for the next 12 hours in medium with 0% FCS and stimulated for 24 hours with TF-1 cells. After stimulation cells were lysed and analysed for the amount of luciferase protein produced. The mean and SD represent three identical experiments.*

3.2.7 Do TF-1 cells interact with MS-5 cells via HA?

The response of MS-5 cells to TF-1 cells is by and large similar to that obtained with HA. Moreover, TF-1 cells can bind HA on their surface (Fig.35). Therefore we tested whether HA might be also involved in the TF-1 mediated response. To remove HA from the reaction, cells were pre-treated with HA'ase prior to addition to the MS-5 cells. HA'ase treatment did not affect TF-1 mediated cytokine response (Fig.36).

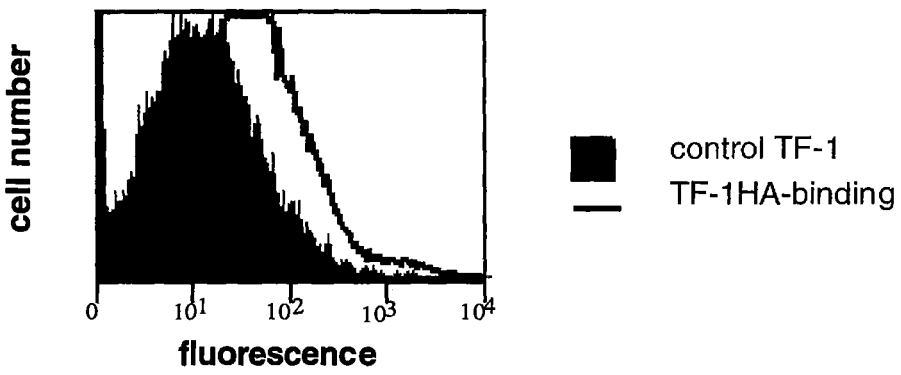


Figure 35. TF-1 cells bind HA (FACS staining with FITC-HA). *10^5 cells were stained with FITC labelled HA. The fluorescence was analysed by FACS-Star plus flow cytometer.*

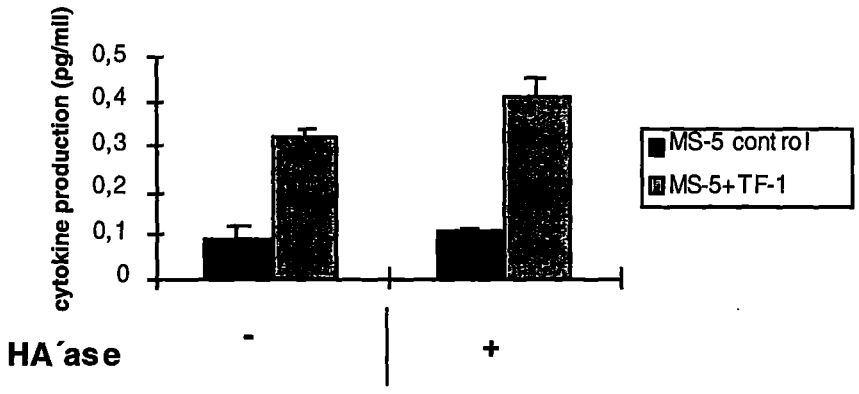


Figure 36. The HA'ase treatment does not abrogate TF-1 mediated cytokine response. *MS-5 cells were grown up to confluence. 10^5 TF-1 were co-cultured on MS-5 cells with or without HA'ase pre-treatment (HA'ase was present during whole incubation time). Cytokine concentration in the supernatants was measured by an ELISA kit. SD was calculated from results obtained from triplicates.*

One still cannot completely exclude a role of HA in this process. Firstly, the components in HA'ase preparation itself can non -specifically stimulate MS-5 cells (data not shown).
Secondly, MS-5 cells might be stimulated by smaller HA products which were not removed from the reaction. However, we believe that the TF-1 mediated and HA mediated induction of cytokines in the MS-5 stromal cell line are two independent events.

Taken together, our findings indicate that MS-5 stromal cells can be induced by HA to produce the cytokines IL-6, GM-CSF and IL-4. The production of the IL-6 cytokine is regulated by a pathway involving Raf, MEK and ERK molecules. This signal cascade results in the upregulation of the IL-6 expression on the transcriptional level through the NF-κB and the NF-IL-6 cis-elements in the IL-6 promoter.
The MS-5 cell line can also be stimulated to produce the same cytokines by direct contact with progenitor cells. In this case the mechanism of IL-6 activation also involves NF-κB and NF-IL-6 elements in the IL-6 promoter.

4 Discussion

Close contact with the bone marrow microenvironment is an essential requirement for the maturation of hematopoietic cells in adult mammals. In this work we concentrated on two components of the marrow environment: the stromal cells and the ECM component HA. We have chosen the murine hematopoietic supportive cell line MS-5 as an experimental system to gain insight into the mechanisms regulating HA-triggered cytokine production in stromal cells.

At first we investigated the cytokine profile induced by HA in the MS-5 cell line. We observed the production of IL-6, GM-CSF and IL-4 upon HA stimulation. We showed that despite the fact that MS-5 cells bind HA via CD44, the pre-treatment of the MS-5 cells with anti-CD44 KM81 mAbs, that block HA binding does not affect IL-6 nor GM-CSF production.

HA induces a signalling cascade in MS-5 cells which results in the activation of the ERK and p38 MAPKs pathways as well as in cytoskeleton rearrangement. Using a Raf-1 dominant-negative mutant we proved that Raf is an upstream target in the HA-induced signalling cascade.

HA induced production of IL-6 is regulated on the transcriptional level, whereas the increase of GM-CSF is due to RNA stabilisation. NF-κB and NF-IL-6 cis-sequences in the IL-6 promoter are the targets of HA action.

The progenitor cell line TF-1 induces the production of IL-6, GM-CSF and IL-4 cytokines in MS-5 cells as well. Furthermore, TF-1 cells also trigger IL-6 and GM-CSF response in a CD44 independent manner. The induction of IL-6 by TF-1 cells is regulated on the transcriptional level and is mediated by NF-IL-6 and NF-κB cis-elements in the IL-6 promoter.

4.1 HA-triggered cytokine activation in MS5 cells.

The involvement of ECM components, especially HA, in hematopoiesis and cytokine regulation was shown previously. HA can stimulate several cells of hematopoietic origin such as macrophages and dendritic cells to produce various cytokines (McKee *et al.*, 1996; Hodge-Dufour *et al.*, 1997; Haegel-Kronenberger *et al.*, 1998). In LTBMC the HA-triggered IL-6 production

seemed to be the major effector of hematopoiesis (Khaldoyanidi *et al.*, 1999).

In MS-5 cells HA upregulates the production of IL-6, IL-4 and GM-CSF. HA- triggered IL-6 and GM-CSF stimulation was already described in BMDM and dendritic cells. The induction of IL-4 upon HA-stimulation is reported here for the first time.

With respect to hematopoiesis, those cytokines have different target cells and act at distinct steps of the hematopoietic process. IL-6 as described before (see introduction) has a direct effect on primitive hematopoietic progenitor cell proliferation and maintenance, and synergistically interacts with other cytokines to stimulate the myeloid proliferation (Suda *et al.*, 1988). In addition, IL-6 stimulates the granulocyte-macrophage and megakaryocyte colony formation (Lotem *et al.*, 1989; Jansen *et al.*, 1992). GM-CSF stimulates the proliferation of marrow progenitors including granulocytes, macrophages, erythrocytes (for review see Metcalf, 1986). It effectively induces granulocyte and monocyte differentiation and synergises with erythropoietin to support the growth of erythroid burst forming units (Sieff *et al.*, 1985). Later on, GM-CSF enhances the functions of mature effector cells (Handman *and* Burges, 1979; Weisbart *et al.*, 1986; Grabstein *et al.*, 1986; Weiser *et al.*, 1987). IL-4 has a more restricted effect in the hematopoietic system and was described to act predominantly in lymphopoiesis. It stimulates B-cell maturation and immunoglobulin production; generates cytotoxic and helper T-lymphocytes (Howard *et al.*, 1982; Vitetta *et al.*, 1984; Noma *et al.*, 1986; Coffman *et al.*, 1986). By interacting with other growth factors it takes part in the regulation of megakaryocyte, granulocyte-macrophage and mast cell proliferation in murine system (Peschel *et al.*, 1987; Broxmeyer *and* Cooper, 1988). Taken together these data indicate that the induction of those three cytokines by the same population of bone marrow stromal cells might be required to support different sub-populations of progenitor cells.

The cytokines induced by HA in MS-5 cells are in part different from those induced in BMDM, where IL-1β and IL-6 were upregulated upon HA-treatment. A plausible explanation for this fact could be that this difference reflects a divergent action of HA on various stromal cell compartments. Most

importantly, the IL-6 cytokine, which was shown to be the crucial component induced in LTBMC, was also produced in MS-5 cells upon HA stimulation. However, the LTBMC cultures mimic some (particularly early) but not all steps of hematopoiesis. Therefore, production of two other cytokines (particularly GM-CSF) can also be physiologically relevant for other (or later) steps in hematopoiesis or for certain sub-populations of hematopoietic progenitors which are not present in LTBMC.

4.1.1 Putative receptor for the HA effect in MS5 cells.

The HA effect on the MS-5 cells could be mediated in two different ways: HA can be uptaken via endocytosis or may act through cell surface HA-receptors. We believe that in our system an interaction with a receptor is most likely involved. Indeed, MS-5 cells could be induced by immobilised HA from HA-covered plates as well as by soluble HA. Unfortunately, we could not support this observation by using endocytosis inhibitors, since they dramatically affected the morphology and survival of MS-5 cells, therefore we cannot completely exclude the possibility of an endocytotic process.

The possible involvement of a receptor —ligand interaction raises the question about the relevant HA-receptor responsible for the effect. One candidate could be the CD44 adhesion molecule. CD44 is known to be the principle receptor for HA and has been shown to be involved in the regulation of hematopoiesis (Aruffo *et al.*, 1990; Moll *et al.*, 1998). Moreover, HA binding to CD44 was reported to induce IGF-1, IL-1β, MIP-1α, MIP-1β and TNF-α cytokines in BMDM (Noble *et al.*, 1993; McKee *et al.*,1996). Recently, HA-induced IL-6 production by dendritic cells was reported to be CD44 mediated (Haegel-Kronenberger *et al.*, 1998). Although the HA binding by MS-5 cells could be by and large repressed by anti-CD44 KM81 mAbs, the same mAbs did not abrogate IL-6 increase nor the GM-CSF production suggesting the action of HA via a different receptor. This observation is in agreement with previous findings in BMDM, in which IL-6 production was also induced in a CD44 independent manner (Khaldoyanidi *et al.*, 1999).

There are several evidences that other unknown HA-receptors are existing. Moreover, HA effects can be mediated via such HA-binding receptors, even

when CD44 is also present on the cell surface. For example it was shown that brain endothelial cells carry an unknown HA-binding receptor in addition to CD44 (Rahmanian *et al.*, 1997). The stimulating effect of HA on BMDM is provided by CD44 and another unidentified receptor (Khaldoyanidi *et al.*, 1999). HA-mediated chemokine induction in alveolar macrophages, described by McKee et al, is only in part mediated by CD44 and the existence of additional unknown receptor was postulated by the authors (McKee *et al.*, 1996).

The possible presence of an HA binding protein, other than CD44, on MS-5 cells is supported by CPC precipitation experiments, where several proteins can be shown to interact with HA. Taken together, we propose that MS-5 cells carry in addition to CD44 another (or others) HA-binding receptor(s) which might be responsible for the HA-mediated cytokine upregulation.

4.1.2 HA-induced signalling cascade

The interaction between HA and cell surface receptors has been shown to generate a variety of signalling events including triggering of tyrosine kinase activation, transient increase of tyrosine phosphorylation of some proteins, and focal adhesion turnover (for review see Entwistle *et al.*, 1996). In MS-5 cells we observed activation of p38 and ERK MAPKs upon HA stimulation. In BMDMs, HA also induced activation of ERK and p38. However, in BMDM the p38 activation was involved in the induction of IL-1β. In our system p38 activation did not result in IL-1β upregulation. One could speculate that the signalling cascade induced by HA is similar between different stromal cells. However, it might lead to the regulation of divergent target genes underlining additionally the divergence between stromal cell populations.

In addition to MAPK activation we observed cytoskeleton rearrangement in MS-5 cells upon HA treatment leading to lamellopodia- like filament structures and ruffles. The signalling pathways that induce different cytoskeleton patterns converge at one or several members of the Rho family of small GTP-binding proteins (Cdc 42, Rac and Rho) (Zigmond, 1996). As it was described in a fibroblast model, constitutively active Cdc42 induces lamellopodias, activation of Rac leads to the membrane ruffles, and

activation of Rho induces stress fibers (Nobes *and* Hall, 1995). Therefore the induction of lamellopodias and ruffles in MS-5 cells upon HA treatment would speak for an activation of Cdc 42 and Rac GTP-binding proteins.

4.1.3 HA-induced signal regulates IL-6 production on the transcriptional level

We found that in MS-5 stromal cells the IL-6 production is regulated on transcriptional level, whereas the GM-CSF response seems to be controlled by posttranscriptional events. This is in line with the fact that the regulation of IL-6 gene in most systems occurs at the transcriptional level although mechanisms of post-transcriptional regulation have also been described (Akira *and* Kishimoto, 1997). For example activation of IL-6 by IFN-γ and TNF-α is transcriptionally controlled. Induction of IL-6 by hypoxia, by human hepatitis B virus, LPS and a variety of other agents as well as repression by the estrogen receptor or estradiol is also regulated at the transcriptional level (Ray *et al.*, 1995; Matsiu *et al.*, 1999; Ohno *et al.*, 1999). GM-CSF can be regulated in both transcriptional and postranscriptional manner depending from the inducing event as it has been described for bone marrow stromal cells (Derigs *et al.*, 1994). Interestingly, in MS-5 cells the same stimulus (HA) controls the production of GM-CSF and IL-6 on different levels.

The observation that IL-6 is induced by HA on the transcriptional level means that the interaction between certain transcription factors and corresponding DNA cis-elements is responsible for this phenomena. Several cis-elements controlling IL-6 expression were previously described in the IL-6 promoter. We report here that NF-κB and NF-IL-6 cis-elements are crucial for the HA-mediated IL-6 response. Our finding that NF-κB is involved in HA response is in agreement with the case of the INOX gene induced by HA in monocytes, where NF-κB activation is also needed (McKee *et al.*, 1997). To our knowledge, we report here for the first time the involvement of NF-IL-6 in a response to a component of the ECM.

4.1.4 NF-κB and NF-IL-6 factors are the targets for HA-signalling

4.1.4.1 NF-κB

NF-κB exists in the cytoplasm of a majority of cell types as homo- or hetero-dimers of a family of structurally related proteins (Rel-family). In its inactive form NF-κB is associated with the inhibitory protein (IκB) and can be activated in cells by a wide variety of stimuli through the signal-induced proteolytic degradation of IκB in the cytoplasm (Fig.37).

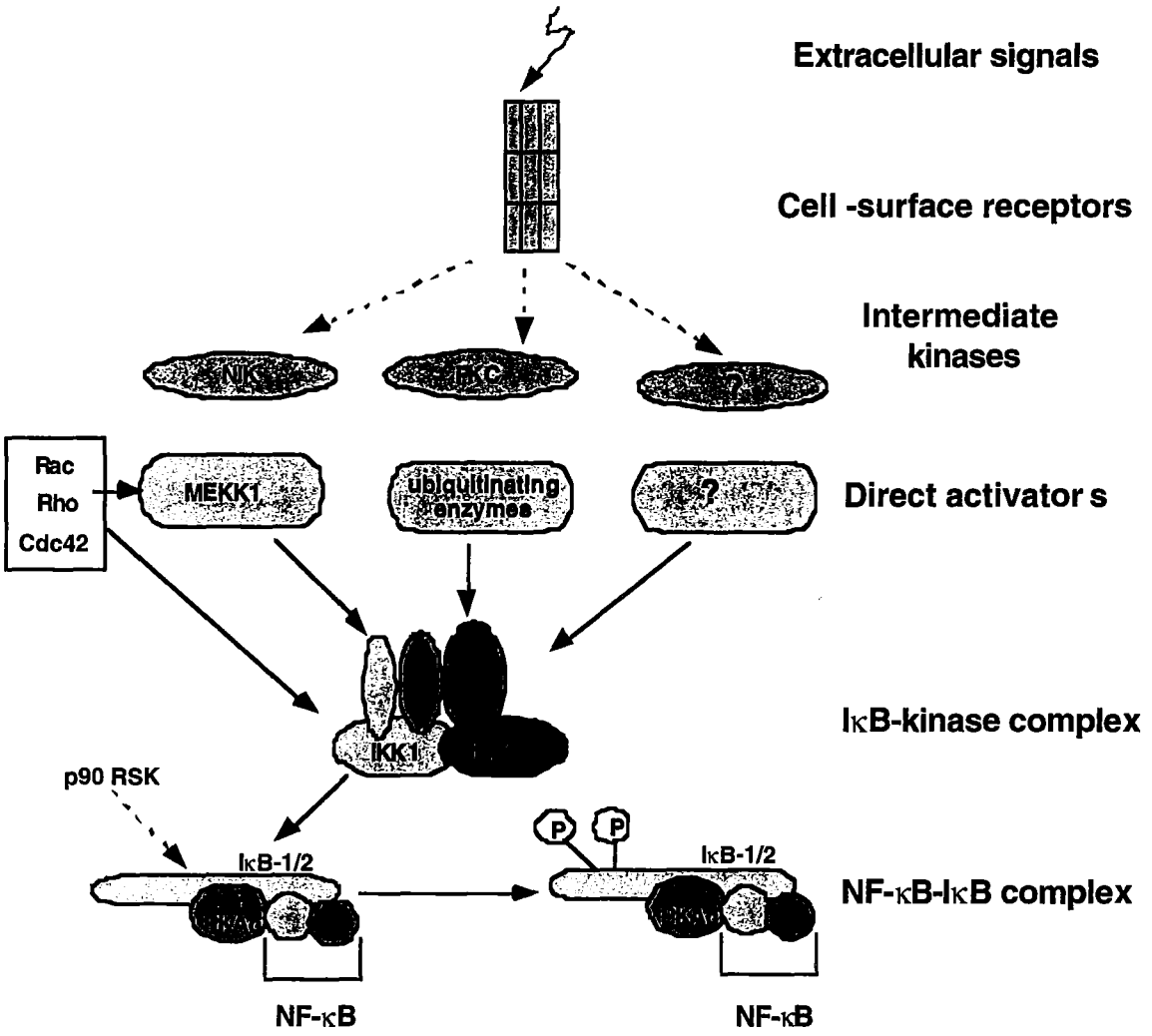


Figure 37. Signalling cascades leading to the activation of NF-κB. ***IKKα/IKKβ:*** *IκB Kinase;* ***MEKK1:*** *Mitogen- activated protein kinase/ extracellular signal-regulated kinase kinase-1;* ***NIK:*** *NF-κB inducing kinase;* ***p90-RSK:*** *p90-ribosomal s6 protein kinase;* ***P:*** *phosphate;* ***PKAc:*** *Protein kinase A catalytic subunit;* ***PKC:*** *protein kinase C.*

With regard to the HA-mediated IL-6 production, we have been able to show two important points. Firstly, the removal of NF-κB cis-element from the IL-6 promoter completely abolishes HA-induced IL-6 response. Secondly, the inhibition of the Raf- Mek -Erk pathway abrogates HA-mediated IL-6 production. These findings suggest a putative connection between the ERK cascade and the NF-κB activation in our model.

Several observations from other systems support our findings. E.g. in lymphoblastoid cells overexpression of either MEK1 or ERK results in constitutive nuclear localisation of NF-κB (Briant *et al.,* 1998). In COS cells the downstream kinase of Raf-MEK-ERK pathway -90 RSK1- has been observed to phosphorylate the N-terminal regulatory domain of IκB and thus to enhance NF-κB DNA-binding (Ghoda *et al.,* 1997). Okadaik acid-stimulated IL-6 production in human monocytes includes the activation of Raf-MEK-ERK cascade resulting in enhancing of the transactivation capacity of NF-κB (Tuyt *et al.,* 1999). In addition, okadaic acid-activated ERK was reported to phosphorylate GST-IκB *in vitro* (Sonoda *et al.,* 1997).

These findings suggest the involvement of the classical ERK pathway in the inactivation of IκB. Our observations provide additional evidence that the ERK pathway might be a signalling pathway regulating the NF-κB activity, however the mechanism of this activation is still unclear.

The p38 activation was also shown to result in NF-κB activation in some systems (reviewed by Schulze-Osthoff *et al.,* 1997; Vanden Berghe *et al.,* 1998). Although we did not test the direct involvement of p38 signalling pathway in HA-triggered NF-κB activation in our system, we believe that p38 activation is not relevant in this respect. The basis for this assumption is that a specific p38 inhibitor does not affect HA-induced IL-6 response.

4.1.4.2 NF-IL-6

The NF-IL-6 transcription factor, also named CEBP/β, is a member of the CCAAT/enhancer-binding proteins (CEBP) family. This factor modulates expression of multiple genes important in host adaptive-, immune- and hematopoietic processes.

NF-IL-6 is responsible for the activation of IL-6 by several stimuli including IL-1, IL-4, bacterial and viral infections (Nakazato *et al.,* 1998; Zhu *et al.,*

1996; Zhang *et al.,* 1995). We show in this work involvement of NF-IL-6 in HA-triggered IL6- response.

NF-IL-6 activity is regulated by phosphorylation and can be achieved by different mechanisms. Of particular interest is the finding that the Ras-Raf-MEK-ERK pathway leads to phosphorylation and activation of NF-IL-6 (Popik *et al.,* 1998; Reddy *et al.,* 2000). This is in accordance with our assumption that the HA signal leading to NF-IL-6 activation might include and require stimulation of the Raf-MEK-ERK cascade.

4.1.4.3 NF-κB and NF-IL-6 synergise in HA-induced IL-6 induction

In our experimental system NF-IL-6 factor seems to synergise with NF-κB.

A number of different transcription factors have been reported to be able physically and functionally to interact with C/EBP members and in particular with NF-IL-6. The interactions with members of NF-κB family of transcription factors play a crucial role in the regulation of expression of various cytokine genes (Yamanaka *et al.,* 1998; Akira *and* Kishimoto, 1997). Synergistic activation by NF-IL-6 and NF-κB has been also shown for the regulation of the genes encoding the acute phase response (Matsusaka et al., 1993). The induction of IL-6 by hypoxia was also shown to be mediated by NF-κB and NF-IL-6 factors in monocytes (Matsui *et al.,* 1999). Synergism between NF-κB and NF-IL-6 factors is also responsible for IL-6 regulation by estrogenes (Stein *et al.,* 1995).

In our experiments, skipping or mutating the NF-κB cis-element resulted in complete abrogation of HA-induced IL-6 production, whereas skipping or inactivation of the NF-IL-6 element showed some residual HA-mediated IL-6 response. A similar effect was observed in hypoxia regulated IL-6 expression (Matsui *et al.,* 1999). One could assume that NF-κB cis-element suffice for the initial induction, but the promoter activation needs a subsequent accumulation of a certain amount of transcription factors.

We speculate that since the induction of NF-IL-6 usually occurs relatively late after stimulation, it is likely that the transcription factor NF-κB whose activation is normally much faster but transient is responsible for the initiating of induction of IL-6, being accompanied later on by NF-IL-6 members.

4.2 TF1- MS-5 cross-talk

4.2.1 MS-5 cell line supports the growth of TF-1 cells

MS-5 cells support survival and proliferation of the progenitor cell line TF-1. During the preparation of this work a paper was published which also reports the support of TF-1 cells by the MS-5 cell line (Heberlein *et al.*, 1999). The authors implied that the Stem cell factor (SCF) produced by MS5 cells is responsible for this supportive effect. The cytokine GM-CSF was not considered as a supportive factor by these authors. We, as well as other groups (Suzuki *et al.*, 1992), showed that MS-5 cells produce a basic level of GM-CSF and IL-6 and the amount of GM-CSF and IL-6 is significantly upregulated upon TF-1 co-culture. In addition mouse pure recombinant GM-CSF also supports the proliferation of TF-1 cells. Moreover, it was found that IL-6 as well support the growth of TF-1 cells (Kitamura *et al.*, 1991). Taken together, these data suggest that in addition to SCF, GM-CSF and IL-6 might be responsible for the MS-5 effect on TF-1 cells.

4.2.2 Progenitor- mediated cytokine induction in stromal cell

The cytokines induced by TF-1 cells in MS-5 cells are the same as those induced by HA treatment. Furthermore, TF-1 cells regulate IL-6 production in MS-5 cells at the transcriptional level through NF-κB and NF-IL-6 cis-elements similar to HA. Multiple myeloma cells can also trigger NF-κB activation and IL-6 production in stromal cells (Chauhan *et al.*, 1996). Contact between thymocytes and thymic epithelial cells (TEC) activates NF-IL-6 and NF-κB transcription factors as well as IL-6 production in human TECs (Ramarli *et al.*, 1998). These observations argue for the general relevance of this cytokine induction in stromal cells by progenitor cells in the hematopoietic system. Therefore, we can assume that this cytokine induction is rather a more common feature of progenitors than just a unique ability of TF-1 cells.

4.2.3 How does TF-1 activate MS-5 cells?

Two possibilities can be proposed for TF-1 action on stromal cells. They can either stimulate stromal cells via cell- cell contact or upon production of a soluble MS-5 activating factor. Recently the upregulation of IL-6 and G-CSF in human bone marrow stromal cell layers upon co-culture with $CD34^+$ bone marrow cells in LTBMC was reported (Gupta *et al.,* 1998b). In this report the authors postulated the production of a soluble factor by progenitor cells, since direct contact between cells appeared not to be essential in that system. In our experimental system the contact between progenitors and stromal cells is required for the upregulation of cytokine production, since the separation of cells by a transwell membrane or pre-treatment of cells with suramin could abolish the TF-1 mediated cytokine response.

We did not clarify yet which receptor could be responsible for the TF-1 induced cytokine production in MS-5 cells, although we showed that this effect is independent from CD44 adhesion receptor. Most likely candidates are integrins, which are well known to play a role in cell -cell adhesion and cytoskeleton rearrangement (Clark *et al.,* 1998; Defilippi *et al.,* 1999). It has been already shown that integrins play a role in adhesion of progenitor cells to the stromal cells (Simmons *et al.,* 1992). Furthermore, several reports implicated integrin family members in cytokine induction (Yurochko *et al.,* 1992; Jarvis *et al.,* 1995; Lee *et al.,* 1999). Moreover, there are observations connecting integrin-induced signals to NF-κB activation (Udagwa *et al.,* 1996; Rosales *and* Juliano, 1996; Scatena *et al.,* 1998). Lastly, monoclonal antibody-mediated clustering of α3β1 or α6β4 integrins have been shown to mimic thymocyte contact and to activate interleukin-6 production in human thymic epithelial cells by the same mechanism as the one used by thymocytes (Ramarli *et al.,* 1998).

The similarity between HA and TF-1 mediated MS-5 activation triggers the question whether TF-1 cells might act on MS-5 cells via HA. Since the treatment of TF-1 and MS-5 cells with hyaluronidase could not affect TF-1 mediated cytokine response, we assume that despite some similarities, TF-1 and HA action on stromal cells might represent two independent mechanisms. This conclusion is in line with experiments in thymocyte or multiple myeloma models where no intermediate ECM components were

shown to be involved in cytokine production in stromal cells upon cell-cell interactions (Chauhan *et al.,* 1996; Ramarli *et al.,* 1998).

In conclusions, this work explored the mechanism of regulated cytokine production by stromal cells. We were able to show several important points:

1. HA and TF-1 cells activate cytokine production in the stromal MS-5 cells.
2. IL-6 is transcriptionally upregulated.
3. The signal cascade to IL-6 gene activation involves the RAF-MEK- ERK pathway.
4. HA and $CD34^+$ TF-1 progenitors regulate IL-6 expression via NF-kB and NF-IL-6 DNA- cis-elements.
5. Despite some similarities, the HA-induced and TF-1 induced cytokine production seem to present two independent mechanisms.

Abbreviations

A	Ampere
ACT D	Actinomycin D
AGM	Aorta- Gonad-Mesonephros
APS	Ammonium persulfate
ATP	Adenosine Triphosphate
BMDM	Bone Marrow Derived Macrophages
BMP	Bone Morphogenic Protein
bp	Base pair
Bq	Bequerel
BSA	Bovine Serum Albumin
CAM	Cell Adhesion Molecule
CD	Cluster of Differentiation
cDNA	Complementary DNA
Ci	Curi
cm	Centimeter
CPC	Cetylpyridinium chloride
cpm	Counts per minute
CS	Chondroitin sulfate
CSF	Colony Stimulating Factor
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
Dom-neg	Dominant negative
DTT	Dithiotreitol
EB	Embryonic Body
ECL	Enhanced chemiluminescence
ECM	Extracellular Matrix
Eds	Editors
EDTA	Ethylendiamine-N,N-tetracetate
ELISA	Enzyme- linked immunosorbent assay
ERK	Extracellular signal regulated kinase
ES cell	Embryonic Stem Cell
et al	and others (Lat. et alii)
FACS	Fluorescence- Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocianate

g	Gram
GAG	Glycosaminoglycan
G-CSF	Granulocyte- Colony Stimulating Factor
GFP	Green Fluorescence Protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
h	Hour
HA	Hyaluronic acid
HEPES	N-2-Hydroxyethylpiperrasine-N'-2-ethansulfonic acid
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish peroxidase
HSC	Hematopoietic Stem Cell
HSP	Heparan sulfated proteoglycan
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IL	Interleukin
INOS	Inducible nitric oxide synthase
K	Kilo (10^3)
Kb	Kilobase (1kb=1000bp)
kDa	Kilodalton (1kd=1000daltons)
L	Litre
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharide
LTBMC	Long Term Bone Marrow Culture
Luc	Luciferase
m	Milli (10^{-3})
μ	Micro(10^{-6})
M	Molar
MAb	Monoclonal antibody
MAPK	Mitogen- activated protein kinase
M-CSF	Macrophage- Colony Stimulating Factor
min	Minute
MIP	Macrophage Inflammatory Protein
MME	Matrixmetalloelastase
MMP	Matrixmetalloproteinases
MOPS	4-morpholinepropanesulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
n	Nano (10^{-9})
NF-IL-6	Nuclear factor IL-6
NF-κB	Nuclear factor-κB

NP-40	Nonidet P40
O/N	Overnight
°C	Degree Celsius
OD	Optical density
p	Pico(10^{-12})
PAGE	Polyacrilamide gel electrophoresis
PAS	Paraaortic-Splanchnopleura
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PI3K	Phosphatidyl-Inositol-3 Kinase
PMSF	Phenylmethylsulfonyl fluoride
R	Recombinant
Rel	Relative
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Room Temperature
RT-PCR	Reverse transcription PCR
SCF	Stem Cell Factor
SD	Standard deviation
SDS	Sodium dodecil sulfate
sec	Second
st	Standard
TEC	Thymic Epithelial Cell
TEMED	N,N,N',N'-tetramethyl-ethylendiamin
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	Tris-(hydroxymethyl)- aminomethan
tRNA	transfer RNA
U	Unit
UV	Ultraviolet
v	Variant
V	Volt
v/v	Volume per volume
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VLA	Very Late Antigen
vol	Volume
W	Watt
w/v	Weight per volume
wt	Wild type

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