MOLECULAR CHARACTERIZATION OF TRANSFORMING GROWTH FACTOR-β3

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MOLECULAR CHARACTERIZATION OF TRANSFORMING GROWTH FACTOR-β3

Proefschrift
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op gezag van de rector magnificus,
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1. Er zijn goede indirecte bewijzen dat transforming growth factor-β3 een multifuctioneel gedrag vertoont *in vivo*.

2. De afname van de TGF-β productie door estrogeen vindt niet noodzakelijkerwijs plaats op het post-transcriptionele niveau zoals geconcludeerd werd door Knabbe *et al.* (1987), maar kan worden verklaard door de aanwezigheid van verscheidene genen voor TGF-β iso-vormen, die verschillen in hun transcriptionele regulatie.
   Knabbe *et al.*, Cell *48*, 417-428 (1987).

3. De waarneming van Arrick *et al.* (1990) waarin de MCF-7 borstkankercellen geen TGF-β2 en TGF-β3 mRNA expressie vertonen, is mogelijk te wijten aan de aanwezigheid van estrogeen in het serum en het estrogene effect van fenolrood in de media gedurende de celgroei.
   Arrick *et al.*, Cancer Res. *50*, 299-303 (1990).
   M.-H. Jeng, pers. communicatie.

4. Bepalingen van de juiste relatieve biologische activiteit tussen de verschillende TGF-β iso-vormen voor een bepaald celtype dienen te worden uitgevoerd onder α2-macroglobuline-vrije condities in het groeimedium.
   Danielpour en Sporn, J. Biol. Chem. *265*, 6973-6977 (1990).

5. De activine iso-vormen spelen zeer waarschijnlijk een belangrijke rol in de inductie van mesoderm gedurende de gewervelde embryogenese.
   Smith *et al.*, Nature *345*, 729-731 (1990).
   Van der Eijnden-Van Raaij *et al.*, Nature *345*, 732-734 (1990).
   Thomson *et al.*, Cell *63*, 485-493 (1990).

6. De door Pribilla *et al.* gedeeltelijk gezuiverde hittebestendige remmer van protein kinase C is het EDTA, dat was toegevoegd in de homogenisatie buffer.
   Pribilla *et al.*, Eur. J. Biochem. *177*, 657-662 (1988).
   Zevgolis *et al.*, Eur. J. Biochem. *188*, 203-204 (1990).

7. Adjuvant therapie met tamoxifen zou mogelijk van voordeel kunnen zijn voor estrogene-receptor negatieve borstkankerpatienten.
   Breast Cancer Trials Committee, Lancet 2, 171-175 (1987).

8. De expressie van de NM23 gen blijkt geassociëerd te zijn met een goede prognose in geval van borstkanker.
   Bevilacqua *et al.*, Cancer Res. *49*, 5185-5190 (1989).
   Hennessey *et al.*, J. Natl. Inst. *83*, 281-286 (1991).

9. Als bij een rechtzaak de rechtspleging gebaat is bij een getuigenis, die gebruik maakt van "DNA finger printing", is een kritische wetenschappelijke inspectie van het bewijsmateriaal noodzakelijk.
   Neufeld and Colman, Scientific Am. *262*, 46-653 (1990).

10. In studies waarin de bevindingen direct de volksgezondheid betreffen is het van groot belang dat de onderzoekers hun resultaten, voordat deze aan de pers worden medegedeeld, laten beoordelen door collega's.

11. Er kan een belangenconflict ontstaan wanneer klinische onderzoekers, die de toxicologie en effectiviteit van nieuwe medicijnen evalueren als betaling effecten (opties) ontvangen.

12. In de biotechnologische industrie zijn de beloften voor medische doorbraken en de financiele verwachtingen van investeerders niet altijd in overeenstemming geweest met de technische realiteit.

13. Als onderzoeker denkt men soms eindelijk het licht aan het einde van de tunnel te zien, maar in plaats daarvan blijkt het een tegemoetkomende trein te zijn.

Stellingen behorend bij het proefschrift:
Molecular Characterization of Transforming
Growth Factor-β3

Peter ten Dijke, Wageningen, 27 November 1991.

1. Good circumstantial evidence exists that transforming growth factor-β3 has multi-functional properties *in vivo*.

2. The decrease of the TGF-β production by estrogen does not necessarily occur at the post-transcriptional level as concluded by Knabbe *et al.*(1987), but can be explained by the existance of multiple closely related genes of TGF-βs, that are differently transcriptionally regulated.
   Knabbe *et al*, Cell *48*, 417-428 (1987).

3. The observation by Arrick *et al.* (1990) that MCF-7 breast cancer cells do not express TGF-β2 and TGF-β3 mRNA, is possibly due to the presence of estrogen in the serum and the estrogenic effect of phenol red in the media during cell growth.
   Arrick *et al.*, Cancer Res. *50*, 299-303 (1990).
   M.-H. Jeng, *personal communication*.

4. For true relative potency of the biological effects of the different TGF-β isoforms, the activity measurements should be performed under α2-macroglobulin-free conditions in the media.
   Danielpour and Sporn, J. Biol. Chem. *265*, 6973-6977 (1990).

5. Activins are likely to play a key role in mesoderm induction during vertebrate embryogenesis.
   Smith *et al.*, Nature *345*, 729-731 (1990).
   Van der Eijnden-Van Raaij *et al.*, Nature *345*, 732-734 (1990).
   Thomson *et al.*, Cell *63*, 485-493 (1990).

6. The partially purified heat resistant and low M.W. inhibitor of protein kinase C isolated by Pribilla *et al.* (1988) is the EDTA, that was included in the homogenization buffer.
   Pribilla *et al.*, Eur. J. Biochem. *177*, 657-664 (1988).
   Zevgolis *et al.*, Eur. J. Biochem. *188*, 203-204 (1990).

7. Adjuvant therapy with tamoxifen may be benefitial for estrogen-receptor negative breast cancer patients.
   Breast Cancer Trials Committee, Lancet *2*, 171-175 (1987).

8. The expression of the NM23 gene appears to be associated with good prognosis in breast cancer.
   Bevilacqua *et al.*, Cancer Res. *49*, 5185-5190 (1989).
   Hennessey *et al.*, J. Natl. Inst. *83*, 281-285 (1991).

9. If justice is to be served in courts of law with forensic testimony using DNA finger printing, critical scientific review of the evidence is neccesary.
   Neufeld and Colman, Scientific Am. *262*, 46-53 (1990).

10. In studies in which the findings suggest a direct effect on human health care, the researchers should be particular careful to submit their results to thorough peer review before releasing it to the news media.

11. A conflict of interest is created when clinical investigators, evaluating the toxicity and efficacy of new drugs, are reimbursed by stock(options).

12. In the biotechnology industry the promises for medical break throughs and the financial expectations of investors have not always been in line with the technical realities.

13. As a researcher one sometimes thinks finally to see the light at the end of the tunnel, but instead one is fooled by a train coming towards you.

Propositions that are part of the thesis:
Molecular Characterization of Transforming
Growth Factor-β3.

Peter ten Dijke, Wageningen, 27 November 1991.

Aan mijn ouders
Voor Midory

CONTENTS

Abbreviations

AP	alkaline phosphatase
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
BP	binding protein
BSA	bovine serum albumin
Ca^{++}	calcium ion
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CHO	chinese hamster ovary
CIF	cartilage inducing factor
CMV	cytomegalovirus
CO_2	carbon dioxide
CSF	colony stimulating factor
CTC	cytolytic T-cell
DAG	diacyl glycerol
DB	DNA binding
DCC	deleted in colorectal cancer
dhfr	dihydrofolate reductase
DIA	differentiation inhibitory activity
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
DPP-C	*decapentaplegic* gene complex
EC	embryonal carcinoma
ECDGF	embryonal carcinoma derived growth factor
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ER	estrogen receptor
ES	embryonal stem
FCS	fetal calf serum
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
GAG	glycosaminoglycan
GAP	GTPase activacting protein
G-B	GTP/GDP binding protein
G-CSF	granulocyte-CSF
GM-CSF	granulocyte-macrophage-CSF
hCG	human chorionic gonadotropin
HCl	hydrochloric acid
HER	human EGF receptor
HILDA	human interleukin for DA cells
HLH	helix-loop-helix
HPLC	high performance liquid chromatography
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin

[^{125}I]IdUr	5-[^{125}I]iodo-2'-deoxyuridine
INT	p-iodonitrotetrazolium
IP	inositol phosphate
i.u.	international units
kb	kilobases
kd	dissociation constant
kD	kilo Dalton
KGF	keratinocyte growth factor
LAK	lymphokine activated killer
LIF	leukemia inhibitory factor
M	molar
M-CSF	macrophage-CSF
MEM	minimal essential medium
MIF	mesoderm inducing factor
MIS	mullerian inhibiting substance
MMTV	mouse mammary tumor virus
mRNA	messenger ribonucleic acid
Mtx	methotrexate
MW	molecular weight
NaCl	sodium chloride
NAM	normal amphibian medium
NaOH	sodium hydroxide
NF-I	nuclear factor-I
NGF	nerve growth factor
NK	natural killer
NRK	normal rat kidney
OP-1	osteogenic protein-1
PAGE	*polyacrylamide gel electrophoresis*
PAI-I	plasminogen activator inhibitor I
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PKC	protein kinase C
PK-Ser/Thr	protein-serine/threonine kinase
PK-Tyr	protein-tyrosine kinase
pM	pico Molar
PMSF	phenyl methyl-sulfonyl fluoride
PTH	phenylthiohydantoin
Rb	retinoblastoma
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RSV	rous sarcoma virus
SDS	sodium dodecyl sulphate
SRF	serum response factor
SSC	standard saline citrate
SSV	simian sarcoma virus
TBS	Tris-buffered saline
TFA	trifluoracetic acid
TGF	transforming growth factor
TGI	tissue-derived growth inhibitor

THR	thyroid hormone receptor
TIMP	tissue inhibitor of methalloproteinases
TNF	tumor necrosis factor
TOF	time-of-flight
TPA	12-o-tetradecanoyl phorbol 13-acetate
TR	transcriptional regulator
vol/vol	volume/volume
Vg -1	vegetal protein-1
Vgr -1	*Vg* -1 related gene
wt/vol	weight/volume
WT	Wilms tumor

PREFACE/VOORWOORD

I would like to express my gratitude to all who have contributed to this thesis. A number of people I like to mention by name.

Sincere thanks to Ken Iwata, who started the growth inhibitor project. His knowledge and hands on technical experience in the area of growth factors have been very valuable for the rapid progress that was made. I thank him for his friendship and for taking the time to teach me many techniques.

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I thank Pamela Alia for typing.

I thank Prof. Dr. Ab van Kammen and Dr. Frank Grosveld for giving me the opportunity to obtain a Dutch Ph.D. degree and for their critical reading of the manuscript.

Een speciaal woord van dank aan mijn ouders die mij zoveel gestimuleerd hebben om een academische studie te volgen. Ik draag het proefschrift aan hun op.

Finally, I like to thank Midory, te agradezco el inmenso apoyo recibido en el trabajo y fuera de el.

Peter ten Dijke
Manhasset, December 1990.

CHAPTER ONE

Scope of the Investigation

SCOPE OF THE INVESTIGATION

Normal tissue homeostasis is controlled by a critical balance of positive and negative modulators. Chapter 2 gives an overview of the molecular aspects of growth control, in particular the role of growth factors and oncogene and anti-oncogene products. Uncontrolled growth of cancer cells may result from either an abrogation of growth stimulatory or a deficiency of growth inhibitory pathways. Mediators of growth inhibition include secretory polypeptide growth inhibitors, like transforming growth factor β (TGF-β) and nuclear proteins, like the retinoblastoma gene product. Early studies on organogenesis suggested the presence of growth inhibitors (chalones) to regulate the growth of organs. Postulating that growth inhibitory proteins might have potential in cancer therapy, we began to analyze human tissues for the presence of novel tumor inhibitory factors. Purification of these activities and physico-chemical characterization suggested a relatedness to TGF-β. The biochemistry and cell biology of TGF-β will be reviewed in Chapter 3.

At the start of the investigation, only one TGF-β had been identified. Our subsequent results indicated that a family of TGF-β proteins exists. Conventional purification of these TGF-β-like activities provided only limited quantities of material for analysis. We therefore adopted an alternative strategy which included the isolation of the cDNAs for TGF-β-like factors using TGF-β1 as a probe, assuming that related molecules might possess sufficient sequence similarity to cross-hybridize to a TGF-β1 probe. Differential hybridization of a Southern blot with human genomic DNA probed with TGF-β1 cDNA suggested the presence of a related gene, which we termed TGF-β3. The research described in this thesis includes the molecular cloning and expression of TGF-β3. Furthermore, experiments were carried out to gain insight into the effects of TGF-β3 on cell growth and differentiation and its mechanism of action, including initial studies to gauge the potential therapeutic uses of this factor.

In Chapter 4, we report the cloning of the human TGF-β3 cDNA and the encoded TGF-β3 protein is compared with other members of the TGF-β family. In Chapter 5 the interspecies conservation of TGF-β3 is examined and the chromosomal location of the human TGF-β3 gene is determined. In Chapter 6, the recombinant expression and purification of TGF-β3 is described. The purified TGF-β3 protein has potent growth modulating effects on a number of normal as well as tumor cells. The studies in Chapter 7 were performed to assess the effect of TGF-β3 on osteoblasts and to characterize the specific binding of TGF-β3 to bone cells. TGF-β3 appears to be a potent regulator of functions associated with bone formation. Crosslinking studies showed that TGF-β3 and TGF-β1 associate in a similar fashion with three cell surface binding proteins, which have been characterized as putative receptor types I and II and a membrane-bound proteoglycan, termed betaglycan. The different TGF-β isoforms appear to have different potencies on Mv1Lu mink lung epithelial and fetal bovine heart endothelial cells. In Chapter 8, we investigate the role of TGF-β receptors and serum factors as determinants of the cell-specific responsiveness to the three homodimeric isoforms. The induction of mesoderm in *Xenopus laevis* animal cap explants by TGF-β3 is discussed in Chapter 9. Finally, in Chapter 10 we review the therapeutic applications of growth factors for wound healing.

CHAPTER TWO

Molecular Mechanisms of Cell Growth Control: Role of Peptide Growth Factors and Oncogene Products

MOLECULAR MECHANISMS OF CELL GROWTH CONTROL: ROLE OF GROWTH FACTORS AND ONCOGENE PRODUCTS

Peter ten Dijke

Oncogene Science, Inc., Manhasset, NY, USA

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I. Introduction

Cellular proliferation and differentiation are controlled, in part, by a complex network of interacting growth factors. After cell secretion, growth factors can bind specifically to high affinity cell surface receptors, typically on adjacent cells (paracrine) or on the producer cell itself (autocrine) and exert a local effect (Sporn and Todaro, 1980). Following growth factor-receptor interaction, activated second messengers release intracellular signals, which initiate a complex cascade of responses. Ultimately, these signals lead to the nucleus and change the expression of target genes. Certain genes may be activated or repressed to modulate cell proliferation or differentiation. Other molecules involved in cellular communication (but not discussed in this review) are extracellular matrix proteins, which mediate direct cell-cell contacts (Loewenstein, 1987), steroid and non-steroid hormones, which bind to nuclear receptors(Evans, 1988).

A number of mediators involved in inter- and intra-cellular signaling mechanisms are products of oncogenes (Heldin and Westermark, 1984) or anti-oncogenes (Sager, 1989; Klein, 1987). Oncogenes were first identified in the genome of acutely oncogenic RNA tumor viruses. In 1976, Stehelin *et al.*. showed that a viral transforming gene, or oncogene, was in fact derived from the host cell genome (Stehelin *et al.*, 1976). The normal cellular form of the gene has been termed proto-oncogene. Thus far, more than 50 oncogenes have been identified (Bishop, 1987; Varmus, 1989). Proto-oncogenes can be activated into transforming oncogenes by qualitative or quantitative changes in the protein product. Whereas oncogenes classically act in a growth stimulating fashion, anti-oncogenes appear to have growth inhibitory activities and have been implicated in neoplasia when inactivated (Sager, 1989; Klein 1987). Thus far, only a few have been identified, and their properties will be reviewed. Several oncogenes and anti-oncogenes have been associated with particular forms of cancer (Bishop, 1989).

The complex interactions between inter- and intracellular signaling molecules, oncogenes and anti-oncogenes have been important for our understanding of the molecular pathways regulating normal cell growth, as well as those leading to neoplastic growth. This review will focus on the role of growth factors and their mechanism of action in growth control. Growth factors exhibit multifunctional activities and can participate in a variety of diverse physiological processes, such as embryogenesis, immune response, hematopoiesis, inflammation, tissue repair and remodeling. In this review, however, only the role of growth factors in early mammalian embryogenesis is discussed.

II. Growth Factors

a. Discovery and Nomenclature

Growth factors can be described as a group of small, soluble molecules, usually proteinaceous in nature, that bind to specific high affinity cell surface receptors to regulate cell proliferation and differentiation. Normally, growth factors are present in intracellular spaces at low concentrations and are released from storage upon exposure to the appropriate environmental stimuli. The autocrine and paracrine action of growth factors confines their effect to neighboring cells (Sporn and Todaro, 1980), in contrast with the more classical endocrine hormones which circulate in the blood. However, it has become apparent that this classification is somewhat arbitrary.

Growth factors were initially discovered in the conditioned medium of tumor cells and in tissue extracts. The first to be detected was nerve growth factor (NGF), produced by mouse sarcoma cells and found to stimulate neurite proliferation (Levi-Montalcini and

TABLE 1: Biochemical Properties of Growth Factors

Growth Factor	Structure of Mature Protein: mol.wt.	amino acids	glycosylated	Sources	Target cells and Biological Effects
Epidermal growth factor					
EGF	5.7 kDa	53	no	submaxillary gland, urine	epithelial cells: stimulates proliferation fibroblast cells: stimulates proflieration
TGF-α	5.7 kDa	50	no	embryo, tumor cells	endothelial cells: stimulates proliferation gastrointestinal tract: inhibition of gastric acid secretion
(amphiregulin and pox virus growth factors)					skin: inhibition of hair growth
Transforming growth factor β					
TGF-β1, TGF-β2 and TGF-β3	25 kDa (dimer)	2x112	no	platelets, bone tumor cells	fibroblasts: regulates proliferation, stimulates extracellular matrix formation osteoblasts: regulates proliferation T lymphocytes: inhibits proliferation and differentiation B lymphocytes: inhibits proliferation and immunoglobulin secretion NK cells: cytotoxicity macrophages: inhibits superoxide and cytokine production
Tumor necrosis factor α and β					
TNFα	17 kDa	157	no	activated macrophages, lymphocytes	T and B lymphocytes: stimulates proliferation fibroblasts: stimulates proliferation, prostaglandin release and collagenase release osteoblasts: bone resorption chrondrocytes: cartilage breakdown muscle: proteolysis CNS: fever, sleep
TNFβ	20-25 kDa	169-171	yes		
Nerve growth factor					
NGF	26 kDa	2x118	no	submaxillary gland	neuronal cells: survival and stimulation of differentiation mast cells: proliferation eosinophils and basophils: regulates differentiation

TABLE 1: Biochemical Properties of Growth Factors (Cont'd)

Growth Factor	Structure of Mature Protein: mol.wt.	amino acids	glycosylated	Sources	Target cells and Biological Effects
Fibroblast growth factor					
aFGF	15.5 kDa	140	no	brain, pituitary embryo and tumor cells	many mesenchymal cells: stimulates proliferation
bFGF	16.4 kDa	146	no		
(*hst/ks*, *int*-2, FGF-5, FGF-6, KGF/FGF-7)					
Platelet-derived growth factor					
PDGF-AA, BB and AB	28-35 kDa	124 (A chain)	yes	platelets, tumor cells, activated endothelial cells activated monocyte and macrophages, activated fibroblasts	connective-tissue forming cells: stimulates proliferation monocytes and neutrophils:chemotactic and activating
	(dimer)	140 (B chain)	yes		
Insulin like growth factor					
IGF-1	7 kDa	70	no	extra-embryonic membranes, fetal serum	endothelial cells: chemotaxis chondroblast, myoblasts: stimulates proliferation and differentiation
IGF-2	7 kDa	67	no		osteoblast and neuroblasts: stimulates proliferation and differentiation
Interleukin 1α and 1β					
IL-1α	17 kDa	~159	no	leukemia cells and epithelial cells, connective tissue cells and leukocytes	fibroblasts: proliferation, prostaglandin release, collagenase release T lymphocytes: activation and growth factor release B lymphocytes: proliferation and differentiation osteoblast: bone resorption chondrocytes: cartilage breakdown muscle: proteolysis
IL-1β	17 kDa	~153	no		
Interleukin 2					
IL-2	14-16 kDa	133	yes	T lymphocytes, leukemia cells	T and B lymphocytes: stimulate proliferation and differentiation NK cells: stimulates proliferation macrophages: increased cytotoxicity oligodendrocytes: stimulates proliferation

TABLE 1: Biochemical Properties of Growth Factors (Cont'd)

Growth Factor	Structure of Mature Protein: mol.wt.	amino acids	glycosylated	Sources	Target cells and Biological Effects
Granulocyte colony stimulating factor					
G-CSF	24 kDa	174-177	yes	monocytes	granulocyte, progenitors: stimulates proliferation and differentiation
Macrophage colony stimulating factor					
M-CSF	70-90 kDa (dimer) 40-50 kDa (dimer)	2x223 2x145	yes	fibroblasts	monocytes and macrophages: stimulates proliferation and differentiation
Granulocyte-macrophage colony stimulating factor					
GM-CSF	18-22 kDa	127	yes	activated T lymphocytes, activated fibroblasts and endothelial cells	progenitor cells: stimulates proliferation and differentiaion of granulocytes and macrophages
Interferon α and β					
IFN-α	16-21 kDa	165-172	some	leukocytes (monocytes,	T cells, fibroblasts: growth regulation and activation monocytes/macrophages: growth regulation and activation
INF-β				macrophages and B lymphocytes) fibroblasts	B cells: stimulation of IgG synthesis
Interferon-γ					
INF-γ	20-25 kDa	143	yes	activated T lymphocytes	fibroblasts: regulation of cell growth monocytes/macrophages: activation NK cells, T cells, B cells: activation

TABLE 1: Biochemical Properties of Growth Factors (Cont'd)

Growth Factor	Structure of Mature Protein: mol.wt.	amino acids	glycosylated	Sources	Target cells and Biological Effects
Interleukin 3					
IL-3	20-30 kDa	133	yes	activated T lymphocytes, leukemia cells	stem cells of bone marrow: stimulates growth and differentiation of cell lineages macrophages; enhancement of cytotoxicity
Interleukin 4					
IL-4	20 kDa	129	yes	activated T lymphocytes	T and B lymphocytes: stimulates proliferation and differentiation macrophages: activation
Interleukin 5					
IL-5	50 kDa (dimer)	2x112	yes	activated T lymphocytes	B lymphocytes: stimulates proliferation and maturation, stimulation IgA and IgM production hematopoietic progenitor cells: stimulates growth and terminal differentiation with eosinophils
Interleukin 6					
IL-6	22-29 kDa	184	yes	activated T and B lymphocytes, activated monocyte and fibroblasts keratinocytes and endothelial cells upon culture	B lymphocytes: stimulation of immunoglobulin synthesis T lymphocytes: promotes IL-2 production and differentiation of cytotoxic lymphocytes Pluripotential stem cells: stimulates differentiation hepatocytes: release of acute phase proteins
Interleukin 7					
IL-7	25 kDa	129	yes	bone marrow stromal cells	B and T lymphocytes: stimulates proliferation
Interleukin 8					
IL-8	10 kDa	99	no	fibroblasts endothelial cells	neutrophils: chemotactic T lymphocytes: chemotatic

Hamburger, 1951). Efforts to purify NGF from mouse submaxillary glands which led to the identification of another growth factor termed epidermal growth factor (EGF), which was also present in this tissue. EGF, which stimulates the premature eyelid opening and tooth eruption in newborn mice, was purified using this *in vivo* effect as an assay (Cohen, 1962). Other growth factors such as platelet-derived growth factor (PDGF)(Ross *et al.*, 1974) and transforming growth factor (TGF) were initially detected using *in vitro* assay systems (DeLarco and Todaro, 1978).

Historically, growth factors were named after the biological assay that lead to their identification. For example, to describe growth factors with effects on the immune system, the term interleukin or cytokine is often used, while the name colony stimulating factors (CSFs) has been given to factors that stimulate bone marrow cells to proliferate and differentiate into colonies. However, there is no clear distinction, as many growth factors have been found to be multifunctional, not only regulating growth of multiple cell types, but also affecting differentiation and functional activities (Sporn and Roberts, 1988). Other names, like panregulins, have been proposed for growth factors, but are not widely used (Sporn *et al.*, 1986). Another implication of the multifunctional ability of growth factors is that many growth factors have been and will be rediscovered. Upon isolation and sequence determination of a factor with certain biological activities, it sometimes occurs that the factor has previously been isolated and characterized.

b. Biochemistry of Growth Factors

The structural characteristics and biological effects of a number of growth factors are listed in Table 1. Sequence determination has shown that many are structurally related. EGF is a member of a family of proteins which includes TGF-α (Marquardt *et al.*, 1983), amphiregulin (Shoyab *et al.*, 1989) and poxvirus growth factors (Brown *et al.*, 1985; Chang *et al.*, 1987). These molecules bind to a 170 kD receptor with a protein-tyrosine kinase activity (Downward *et al.*, 1984). Members of this family stimulate proliferation of a large number of cell types including epithelial, fibroblastic and endothelial cells (Nakagawa *et al.*, 1985). The FGF family of growth factors consist of not only of acidic and basic FGF (Abraham *et al.*, 1986; Gimenez-Gallego *et al.*, 1985), but also includes the *hst/ks* (Taira *et al.*, 1987; Dickson and Gordon, 1987) and *int*-2 proto-oncogene products (Smith *et al.*, 1988b), FGF-5 (Zhan *et al.*, 1988), FGF-6 (Marics *et al.*, 1989) and KGF or FGF-7 (Finch *et al.*, 1989). The FGF-family members are mitogenic for cells of mesodermal origin and may bind to multiple receptos types, with different affinities (Gospodarowicz *et al.*, 1986). Three different isoforms of PDGF are formed by homo- or hetero-dimerization between a 124 amino acid A chain and a 140 amino acid B chain (Stroobant and Waterfield, 1984; Heldin *et al.*, 1986; Hammacher *et al.*, 1988; Bowen-Pope *et al.*, 1989). The isoforms PDGF-AA, PDGF-BB and PDGF-AB interact differently with two receptor types, both of which contain an intrinsic protein-tyrosine kinase activity (Hart *et al.*, 1988; Heldin *et al.*, 1988). Three TGF-β homodimeric isoforms are present in humans, which are formed from two polypeptide chains of 112 amino acids sharing approximately 70% amino acid sequence identity (Derynck *et al.*, 1985; de Martin *et al.*, 1987; ten Dijke *et al.*, 1988). All three proteins interact with high affinity, but different potencies, to three cell surface proteins, present on most cells (Cheifetz *et al.*, 1987). Recently, the type I and type II receptors have been implicated in signal transduction of TGF-βs, and appear to account for many, if not all, of the actions of these molecules (Boyd and Massague, 1989; Laiho *et al.*, 1990a). TGF-βs affect the proliferation of multiple cell types and can stimulate as well as inhibit cell proliferation (Roberts and Sporn, 1990). The colony stimulating factors (CSFs) are growth factors for hematopoietic

cells, controlling their survival, proliferation and differentiation (Clark and Kamen, 1987). CSFs are not related structurally, act through different receptors, and are classified according to the type of mature blood cell soft agar colony to which they give rise. Thus, G-CSF and M-CSF support colonies of granulocytes and macrophages, respectively; GM-CSF and multi-CSF (also termed IL-3) stimulate the colony growth of two or more distinct phenotypes. The interleukins act as signaling molecules between the various leucocyte cell types (O'Garra, 1989 a,b). Interleukins also exhibit little structuraly homology to one another.

Different members of a growth factor family with similar *in vitro* activities may have different *in vivo* activities, as different spatial and temporal expression patterns may lead to different physiological functions. Furthermore, *in vitro* sources of growth factors do not necessarily correspond to *in vivo* sources. For example, TGF-β expression is often induced when cells are kept in continuous culture, possibly related to its stimulatory action on extracellular matrix formation which facilitates growth on plastic. Also, the *in vitro* biological effects cannot automatically be translated to *in vivo* activities. TGF-βs inhibit the growth of endothelial cells *in vitro*. However, they are potent angiogenic factors *in vivo*, the probable mechanism being the chemoattraction of macrophages which are activated to secrete angiogenic factors (Roberts *et al.*, 1986).

c. Multiple effects of growth factors

Many growth factors have multiple effects on cell proliferation and differentiation, which appears to be context dependent (Sporn and Roberts, 1988). The presence of other growth factors, the cell type and state of differentiation can all modulate the action of a particular growth factor. A prototype of a multifunctional growth factor is TGF-β. In combination with TGF-α or EGF, TGF-β stimulates the growth of NRK cells in soft agar, whereas TGF-β inhibits the growth of NRK cells in monolayer culture (Anzano *et al.*, 1982). TGF-β stimulates soft agar growth of fibroblasts transfected with a *myc* oncogene, but inhibits growth of these cells in the presence of EGF (Roberts *et al.*, 1985). TGF-β also stimulates cell proliferation of fibroblasts from very early stage fetuses, but inhibits proliferation of fetal fibroblasts from the later gestational stage (Hill *et al.*, 1986).

Growth factors have been suggested to form the signaling components of an intercellular language, whose meaning is controlled by context (Sporn and Roberts, 1988). The amount of information that can be communicated between cells increases enormously when combinations, instead of individual growth factors, are used. However, the detailed mechanisms that result in multifunctional effects and the pathways which allow these complex interactions to be coordinated are poorly understood.

Their multifunctionality, together with their potency (typically in the pico Molar range) clearly necessitate a tight control of their action in a localized region. A number of growth factors are produced in an inactive latent form. For TGF-β, either activation by specific proteases or acidification is necessary to obtain a biologically active protein after secretion (Lawrence *et al.*, 1985; Lyons *et al.*, 1988). A growth factor, following receptor binding, can be inactivated by internalization and degradation by lysosomal proteases (Carpenter, 1987). Alternatively, binding proteins or scavenger proteins, like α2-macroglobulin can inactivate certain growth factors (James, 1990).

III. Growth Factor Receptors

a. Biochemistry of growth factor receptors

Growth factors act through specific cell surface receptors. Radiolabeled biologically active growth factors are often used for labeling of the receptors and to characterize their presence, number and affinity on the cell surface. For purification of the receptor, a growth factor affinity purification step is often employed. As some growth factor receptors have protein tyrosine kinase autophosphorylation activities (Ushiro and Cohen, 1980) affinity chromatography using a phosphotyrosine antibody has also been used in the receptor purification, eg. the PDGF receptor (Yarden, *et al.*, 1986). After purification and partial sequencing or antibody generation, the receptor gene can be cloned by screening cDNA (expression) libraries with oligonucleotide probes or antibodies. Alternatively, direct cloning of the receptor can be achieved by for example, screening cDNA library-transient transfected COS cells with radiolabeled ligand and autoradiography.

Two types of growth factor receptors have been structurally characterized and they can be classified according to the presence or absence of discrete domains. One class of receptors have a characteristic seven membrane spanning motif, such as the β-adrenergic receptors (Dixon *et al.*, 1986) and the *mas* proto-oncogene product (Jackson *et al.*, 1988). The N-terminus and C-terminus, located on the extracellular and intracellular sides of the plasma membrane, respectively, are connected by a polypeptide chain which crosses the membrane seven times. The ligand interacts with the transmembrane helices and extracellular loops, while G-proteins (see also part IV on signal transduction) interact with the intracellular loops and mediate transduction of the extracellular stimulus to the intracellular effector, eg. adenylate cyclase (Sibley *et al.*, 1987). The other class of receptors are single-chain transmembrane glycoproteins, with a glycosylated extracellular ligand binding domain, a hydrophobic transmembrane region, and typically, an intracellular domain which possesses an intrinsic enzymatic activity which directly mediates the intracellular signal production. Within this class different receptor subgroups can be distinguished according to their structural domains and sequence homologies (Ullrich and Schlessinger, 1990).

A number of receptors with a single transmembrane region contain cysteine rich repeats on the external domains, such as the EGF receptor family (EGF-R (Lin *et al.*, 1984; Ullrich *et al.*, 1984), HER-2/*neu* (Bargman *et al.*, 1986), HER-3 (Kraus *et al.*, 1989) and X*mrk* (Wittbrodt *et al.*, 1989)) , with 2 repeats per monomer and the insulin receptor family (I-R (Ullrich *et al.*, 1985), IGF-R (Ullrich *et al.*, 1986) and IRR (Shier and Watt, 1989)) with two repeats per disulfide-linked heterotetramer. A number of receptors contain multiple immunoglobulin like repeats such as the PDGF receptors A and B (Yarden *et al.*, 1987), CSF-1 (Sherr *et al.*, 1985) and the c-*kit* proto-oncogene product (Yarden *et al.*, 1987) which possess 5 repeats, and the FGF receptors, also termed the *flg* and *bek* proto-oncogene products with 3 such repeat structures (Ruta *et al.*, 1989; Pasquale and Singer, 1989). The recently identified hematopoitin receptor superfamily (including the receptors for erythropoietin, IL-2, IL-3, IL-4, IL-6, IL-7, G-CSF, GM-CSF, growth hormone and prolactin) share a characteristic 4 cysteine residues and a Trp-SerX-Trp-Ser sequence motif (Cosman *et al.*, 1990). The TNF receptor shares homology with NGF receptor in their extracellular domains (Schall *et al.*, 1990; Loetscher *et al.*, 1990). The functional significance of the homologous structures is unknown, but suggests that they have evolved from ancestral genes.

Sequence analysis of the cytoplasmic domain has shown that many growth factor receptors posses protein-tyrosine kinase activity, such as EGF-R, IGF-R, PDGF-R and FGF-R, which appears essential for their function (Ullrich and Schlessinger, 1990). Receptors which lack protein tyrosine kinase activity may associate with other proteins having tyrosine kinase activity or other activities.

Some receptors are complexed with oligomeric GTP-regulatory proteins (G-proteins) which serve to couple receptors to intracellular effector molecules (Gilman, 1987). G-proteins are a multi-gene family, composed of α, β and γ subunits. The α-subunit appears to be the active moiety, while the β and γ subunits serve to regulate the α subunit. Receptor stimulation causes the exchange of the bound GDP on the G-protein for GTP. Subsequently, the activated GTP:G-protein complex activates enzymes that generate second messengers such as cAMP. The G-proteins possess an intrinsic GTPase activity (Gibbs *et al.*, 1984), which terminates this activation. Regulatory proteins have been identified that stimulate the GTPase activity, termed GAPs (GTPase activating proteins)(Traheg *et al.*, 1988; Vogel *et al.* 1988).

The number and affinity of receptors is an important control point for signal regulation. The expression of growth factor receptors can be regulated at both the transcriptional and translational level. The affinity of the receptor can also be regulated by post-transcriptional modification leading to conformational receptor changes creating subclasses of receptors. The binding of ligand to the receptor often leads to decreased receptor numbers, a process known as down-modulation (Green and Olefsky, 1982). One mechanism involved in this process appears to be an increased rate of receptor degradation. After binding of the growth factors with their receptors, the complex is internalized and may either be targeted to the lysosomes for degradation (as for EGF) (Carpenter, 1987) or recycled to the cell surface (as for insulin)(Oka *et al.*, 1984). Different receptors are also able to regulate each other, a process known as receptor cross-talk or transmodulation, which involves changes in receptor distribution and affinity between the cell surface membrane and intracellular compartments. For example, addition of PDGF to cells can decrease the affinity of the EGF receptor for its ligand (Bowen-Pope *et al.*, 1983). A detailed description of structure and function of each receptor family is beyond the scope of this review. However, the function and regulation of receptors with protein-tyrosine kinase activity are discussed in more detail.

b. Receptors with Protein-Tyrosine Kinase Activity

Some growth factor receptors possess an intrinsic protein-tyrosine kinase activity (Ullrich and Schlessinger, 1990). Upon ligand binding, the signal is transmitted across the plasma membrane and activates the tyrosine kinase activity of the intracellular domain. The mechanism whereby the signal is transmitted is not clear. One model proposes that growth factors induce dimerization of the receptor (Schlessinger, 1988; Williams, 1989; Heldin and Westermark, 1990). Either the ligand binding may induce a conformational change in the extracellular domains, as shown for the monomeric ligand EGF or the dimeric ligand itself as shown for PDGF, may lead to receptor dimerization. The receptor dimerization presumably stabilizes the interaction of the two cytoplasmic domains with a concomitant activation of the protein tyrosine kinase activity (Yarden and Schlessinger, 1987). Dimerization may also occur between structurally similar receptors leading to hybrid receptors, which could generate different signals. Heterodimerization of the EGF receptor and the p185 ^{neu} protein has been observed to modulate EGF receptor function (Goldman *et al.*, 1990; Wada *et al.*, 1990) The presence of different dimeric ligand isoforms, as is the case for PDGF, can also lead to distinct receptor signaling

(Hammacher *et al.*, 1989). Homology comparison between family members shows that the transmembrane region is less conserved than the extra- and intra-cellular regions, suggesting that it may have only a minor function, if any, in signal transmission. Based on the structure of the transmembrane region it appears unlikely that a conformational signal could be transmitted directly through this region; more likely, this domain functions as an anchor of the receptor to the membrane. It is of interest, however, that a single point mutation in the transmembrane domain of the HER-2/*neu* receptor was found to be sufficient for activation of protein tyrosine kinase function (Stern *et al.*, 1988) and to confer transforming potential to the receptor, possibly by inducing receptor dimerization (Weiner *et al.*, 1989).

IV. Signal Transduction Pathways

a. Cytoplasmic Second Messengers

After a growth factor binds to its receptor, many rapid changes can occur in the cell, including stimulation of inositol formation (Berridge, 1987), Ca^{2+} influx (Moolenaar *et al.*, 1984), activation of Na^+/H^+ exchange (Pouyssegur *et al.*, 1985) and protein phosphorylation of cellular substrates. While there has been an explosion of information in this area in recent years, the events leading to the modulation of gene expression remain poorly understood. It appears that many ligand-receptor systems activate common second messengers. The two best characterized are the cAMP and inositol phosphate pathway. Activation of adenylate cyclase yields cAMP, which in turn activates a cAMP dependent protein kinase (Edelman *et al.*, 1987). Phospholipase C stimulates the hydrolysis of a membrane lipid phosphatidyl inositol 4,5-diphosphate (PIP_2) yielding diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3)(Berridge, 1987). Subsequently, DAG can activate protein kinase C (PKC) and IP_3. IP_3 triggers a transient release of Ca^{2+} from intracellular compartments which elicits a number of rapid metabolic changes. Activation of ion channels, such as the Na^+/H^+ antiporter occur by virtually all growth promoting agents (serum, growth factors, vasopressin, bradykinin, etc). However, it remains controversial if changes in pH and cation concentration act as a second messenger system or merely serve to compensate for pH changes resulting from increased rate of glycolysis.

Recently, it was shown that both PDGF and EGF receptors associate with multiprotein complexes consisting of phosphatidyl inositol kinase (Coughlin *et al.*, 1989) and phospholipase C (Kumjian *et al.*, 1989; Morrison *et al.*, 1990), a GTPase activating protein (GAP)(Kaplan *et al.*, 1990), which is involved in the control of *ras* activity, and the c-*raf* proto-oncogene product, which is a serine/threonine kinase (Kaplan *et al.*, 1990; Morrison *et al.*, 1989) and members of c-*src* family oncogene products (c-*src*, c-*fyn* and c-*yes*) which are tyrosine kinases (Kypta *et al.*, 1990). Upon ligand stimulation these proteins are phosphorylated and activated.

b. Modulation of gene expression

One of the principal mechanisms of growth factor action is to control gene transcription. Ultimately, the transcription of the growth factor target genes is controlled by the interaction of RNA polymerase II with specific transcription factors. These transacting factors recognize and bind specific DNA sequence (cis-acting) elements in the promoter/enhancer region of the gene. The type, number and spacial array of cis elements allows a unique combination of factors to regulate each gene. The organization of the cis elements together with their occupation by the appropriate transacting factors

ultimately determines the transcription rate (Mitchell and Tjian, 1989). Via cytoplasmic second messengers, the status of the transacting factors changes (see discussion below) resulting in either a positive or negative change in the rate of transcription (or activity) of a relatively small number of primary response gene products. Many of these primary response genes themselves encode transcription factors, such as members of the *jun* (c-*jun*, *jun* B and *jun* D)(Maki *et al.*, 1987; Nakabeppu *et al.*, 1988; Ryder *et al.*, 1988) and *fos* proto-oncogene families (c-*fos*, *fra*-1, *fos*-B)(Cohen and Curran, 1988). Their protein products can in turn activate or repress the transcription of secondary response genes, resulting in a cascade of regulated transcriptional modulation.

Growth factors with quite different and multiple biological activities induce the transient expression of a relatively small number of common early response genes (perhaps 50-100)(Herschman, 1989). For example, upon addition of serum, PDGF, EGF or NGF, the expression of the early response gene c-*fos* is rapidly stimulated (Greenberg, and Ziff, 1984; Kruijer *et al.*, 1984; Bravo *et al.*, 1985; Kruijer *et al.*, 1985). A number of potential mechanisms operate to obtain growth factor and cell specific responses. By influencing the number and array of synergizing or antagonizing transcription factors, the transcription rate of different genes will alter differently. Many single promoter elements interact with different structurally related, often dimeric transcription factor complexes (Jones, 1990). Members of the Jun family bind as homodimers or heterodimers with members of the Fos family to a DNA sequence termed the AP-1 binding site (Kouzarides and Ziff, 1989). Dimerization is mediated by the so called basic leucine zipper motif (Landschultz *et al.*, 1988). Different complexes may have different effects on transcription possible by binding to DNA with different affinities or specificities; Jun A, but not Jun B, can activate the transcription from a AP-1 site containing promoter; Jun B can, in fact, decrease the activation mediated by Jun A (Chiu *et al.*, 1989; Schutte *et al.*, 1989). Some transcription factors contain a basic helix-loop-helix (HLH) motif, which also mediates DNA binding and dimerization (Davis *et al.*, 1990). This motif has been identified in two proteins (E12 and E47) that bind to a specific DNA sequence found in immunoglobulin enhancers (Murre *et al.*, 1989) and in a number of genes involved in muscle development (*Myo D*, *Myf* 5 and *myogenin*)(Davis *et al.*, 1987; Braun *et al.*, 1989; Edmonson and Olson, 1989; Wright *et al.*, 1989). Recently, a gene encoding a protein termed Id was discovered and found to contain the HLH motif without the basic DNA binding region (Benezra *et al.*, 1990). The Id protein is able to dimerize with Myo D but fails to bind to DNA, suggesting a role as a dominant negative regulator. The latter mechanism again illustrates the complexity of interactions made possible by using unique combinations of common transcription factors.

The observation that in the presence of protein synthesis inhibitors gene expression can still be regulated suggests that transcription factors are regulated post-translationally; ie. in their phosphorylation or glycosylation states. The c-*jun* proto-oncogene product is phosphorylated after activation of protein kinase C (Angel *et al.*, 1987). The modified transcription factor may be altered in the affinity or in the sequence-specific DNA binding characteristics. Alternatively, the interaction with other proteins in the transcription complex can be affected. This mechanism may be found for the glycosylated p67 serum response factor (SRF) of the *fos* gene, which interacts in a ternary complex with another glycosylated protein (p62 CTF) and the serum response element (SRE) (Herrera *et al.*, 1989; Schroter *et al.*, 1990).

V. Oncogenes and anti-Oncogenes

a. Oncogenes

Oncogenes can be defined as modified cellular (or viral) growth regulatory genes whose altered protein products, acting alone, or more typically in combination with other gene products, can lead to cell transformation. Proto-oncogenes can be activated by qualitative and/or quantitative changes in their gene products. Transduction of proto-oncogenes by acutely transforming retroviruses can result in overexpression, truncation or internal mutations leading to a constitutive biological activity (Bishop, 1987). The transforming protein from the Rous sarcoma virus, pp60 v-*src*, was the first described transduced cellular oncogene (Stehelin *et al.*, 1976). To date, at least 20 distinct cellular genes have been identified through their derivative viral oncogenes present in a retroviral genome. Many exhibit constitutive protein-tyrosine kinase activity (Hunter and Cooper, 1985). Proto-oncogenes can also be activated by point mutation in the coding region, as exemplified by the human *ras* oncogene (Tabin *et al.*, 1982; Santos *et al.*, 1982). Another more frequent activation mechanism is increased expression by direct gene amplification, as found for the N-*myc* gene (Schwab *et al.*, 1984). In animals, an increase in gene expression has also be observed following retroviral insertion whereby the strong transcriptional promoter of the virus stimulates expression of an adjacent proto-oncogene (Nusse and Berns, 1988). The *int*-1 and *int*-2 proto-oncogenes were discovered in such a manner, in association with the mouse mammary tumor virus (MMTV)(Nusse and Varmus, 1982; Peters *et al.*, 1983). Chromosome translocations provides yet another mechanism for activation of proto-oncogenes, the paradigm being the reciprocal translocation of the human c-*abl* proto-oncogene from chromosome 9 to 22 (Groffen *et al.*, 1984). This translocation results in a novel fusion protein with constitutive tyrosine kinase activity.

The finding in 1978 by DeLarco and Todaro that certain tumor cells and oncogene transformed cells often produced their own growth factors (termed transforming growth factors), resulted in an important link between oncogene and growth factor research. This discovery lead Sporn and Todaro to suggest that one mechanism for malignant cell transformation was the ability of the transformed cell to produce and respond to its own growth factors. Support for this hypothesis was obtained with the sequence determination of the v-*sis* oncogene from simian sarcoma virus (SSV), which showed sequence homology to the PDGF B chain (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983), suggesting that growth factors can function as oncogenes.

Subsequently, it was shown that growth factor independence can also be achieved by alterations in the structure of receptors, which can uncouple signal transduction from ligand binding, providing a mitotic signal in the absence of growth factors. The oncogene v-*erb* B, with intrinsic tryosine kinase activity is a truncated version of the EGF receptor (EGF-R), which lacks most of the external EGF binding domain (Downward *et al.*, 1984). The v-*fms* oncogene is a mutated version of the CSF-1 receptor (Sherr *et al.*, 1985). Point mutations induce or stabilize a conformational change that is equivalent to ligand binding (Roussel *et al.*, 1988; Woolford *et al.*, 1988).

Based upon structural similarities and biochemical properties, *ras* proteins presumably function as G-proteins (Hurley *et al.*, 1984). The point mutations reduce the GTPase activity of *ras*, thereby maintaining the *ras* protein longer in its GTP active state. Alterations in the activity of cytoplasmic signal transducers can also lead to a loss of growth control by uncoupling it from the need for exogeneous stimuli. The *src* family of oncogenes (including *yes*, *fgr*, *fps/fes*, *ros* and *abl*) have an intrinsic constitutively active

TABLE 2: Proto-oncogenes and Their Products[a]

Oncogene	Proto-oncogene Product (in kDa)
I. Secreted	
sis	p32/PDGF-B
hst	p22/FGF-like factor
int-2	p27/FGF-like factor
II. Plasma membrane	
a. transmembrane	
erbB	p170/EGF receptor; PK-Tyr
fms	p170/CSF-1 receptor; PK-Tyr
neu	p185/receptor; PK-Tyr
met	p140/receptor; PK-Tyr
ros	p250/receptor; PK-Tyr
kit	p145/receptor; PK-Tyr
trk	p140/receptor; PK-Tyr
ret	p150-170/receptor; PK-Tyr
eck	p130 receptor; PK-T
elk	p130/receptor; PK-Tyr
eph	p130/receptor; PK-Tyr
b. membrane-associated	
H-*ras*	p21: G-B
K-*ras*	p21; G-B
N-*ras*	p21; G-B
src	pp60; PK-Tyr
yes	p61; PK-Tyr
fps/fes	p92; PK-Tyr
abl	p142; PK-Tyr
fgr	p57; PK-Tyr
hck	p57; PK-Tyr
lyk	p56; PK-Tyr
fyn	p60; PK-Tyr
tkl	p50; PK-Tyr
lyn	p58; PK-Tyr

TABLE 2: Proto-oncogenes and Their Products[a] (Cont'd)

Oncogene	Proto-oncogene Product
III. Cytoplasm	
mos	p37; PK-Ser/Thr
raf/mil	p74; PK-Ser/Thr
IV. Nucleus	
myc	p58; TR/DB
fos	p55; TR
jun	p39; TR/DB
erbA	p46; TR/DB/THR
myb	p75; TR/DB
ets	p50; TR/DB
rel	p68; TR/DB
ski	p100; TR?
p53	p53; TR/DB

[a] Abbreviations: (PK-Tyr) protein-tyrosine kinase; (PK-Ser/Thr) protein-serine/threonine kinase; (TR) transcriptional regulator; (DB) DNA binding; (G-B) GTP/GDP binding protein; (THR) thyroid hormone receptor.

TABLE 3: Anti-oncogenes and (Putative) Tumor Suppressor Genes[a]

Anti-oncogene/ Tumor Suppressor	Protein Product and Biochemical Properties
Rb-1 (retinoblastoma gene)	p105; TR/DB
WT-1 (Wilms' tumor gene)	? ; TR/DB
p53	p53; TR?DB
c-*erb*A	p46; TR/DB
K-*rev*	p21; antagonist *ras*
NM23	p17; metastasis inhibitor
Thrombospondin (fragment)	p140; angiogenesis inhibitor
DCC (deleted in colorectal cancer)	? ; adhesion molecule

[a] Abbreviations: (TR) transcriptional regulator; (DB) DNA binding

protein tyrosine kinase activity, providing a continuous intracellular mitogenic stimulus (Hunter, 1989). A number of oncogene products, including *fos*, *myc*, *myb*, *ski* and *jun* are located in the nucleus and are implicated in aberrant control of gene expression or DNA replication (Eisenman, 1989). Thus, proto-oncogenes appear to form the growth stimulating proteins in the signaling pathways in normal growth control (Table 2)(Bishop, 1987).

b. Tumor suppressors and anti-oncogenes

Studies with somatic cell fusions between malignant and normal cells were the first indication that normal cells contained genes that suppressed the malignant phenotype (Stainbridge *et al.*, 1982). Normal human fibroblasts fused with HeLa cells were found to be non-tumorigenic, while revertants of these hybrids had lost one or more chromosomes (Stainbridge *et al.*, 1982). Presumably, in tumor cells both alleles of these tumor suppressing genes (also termed anti-oncogenes) were lost, functionally inactivated or their gene expression reduced. Further support for the existence of tumor suppressor genes came from studies on hereditary predisposition to cancer (Knudson, 1985; Hansen and Cavenee, 1987). Cytogenetic and restriction fragment length polymorphism (RFLP) studies indicated chromosomal deletion or loss of heterozygosity at certain chromosomal regions in tumors, such as chromosome 13 in pediatric retinoblastoma (Cavenee *et al.*, 1983) and chromosome 11 in Wilms tumors (Orkin *et al.*, 1984; Fearon *et al.*, 1984; Koufos *et al.*, 1984). The retinoblastoma (Rb-1) gene was cloned by chromosome walking using RFLP marker for the suppressor gene locus (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). The Rb-1 gene encodes a nuclear phosphoprotein of 105 kDa with affinity for DNA, suggesting an involvement in the regulation of transcription or DNA replication. Defective Rb-1 alleles were not only detected in retinoblastoma, but also in other tumor types, including osteosarcomas (Weichselbaum *et al.*, 1988), small cell lung carcinomas (Harbour *et al.*, 1988) and mammary carcinomas (Lee *et al.*, 1988). Functional evidence for identity of Rb-1 gene was obtained when the wildtype Rb-1 gene was introduced into retinoblastoma and osteosarcoma cells lacking an intact Rb. Upon introduction of Rb-1, cell growth was inhibited *in vitro* and cells lost tumorigenicity *in vivo* (Huang *et al.*, 1988). Recently, a possible candidate for the Wilms Tumor (WT) gene on human chromosome 11 (band 13) was cloned, using similar techniques as used for Rb-1. This WT gene encodes a zinc finger protein, with a potential role in transcriptional regulation (Rose *et al.*, 1990; Call *et al.*, 1990).

To contrast the tumor suppressor genes or anti-oncogenes with the dominant acting oncogenes, the name recessive oncogenes has been proposed. However, recent observations suggest that at least some anti-oncogenes do not act in a recessive fashion. Some familial tumors, such as adenomatous polyposis and multiple endocrine neoplasia type 2 develop without loss of the normal allele. A dosage effect appears to be important with reduction to half the normal dose leading to hyperproliferation (Vogelstein *et al.*,1988; Nelkin *et al.*, 1989; Landsvater *et al.*, 1989).

Recently, two genes, p53 and v-*erb*A, originally classified as oncogenes, appear to act in a dominant negative fashion over their normal counterparts, and have growth suppressive activity. The (anti)oncogene p53 was discovered through the direct association of p53 protein with DNA tumor virus proteins (Lane and Crawford, 1979; Linzer and Levine, 1979). p53 was first shown to immortalize primary cells and collaborate with the *ras* oncogene in transforming of these cells, a characteristic for oncogene function (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). However, a mutated form of p53, rather than wildtype p53, was used in these

experiments. Wildtype p53 lacks any transforming potential and is in fact capable of inhibiting the transforming activity of *ras* and mutant p53 combination or a mixture of *ras* and EIA (Finlay *et al.*, 1989). p53 protein has been shown to oligomerize (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). The dominant negative effect of a large excess of mutant p53 over wildtype may be attributed to the formation of hetero-oligomers, which render the wildtype p53 inactive.

The oncogenes v-*erb*A and v-*erb*B were originally identified as the two cellular genes co-transduced by avian erythroblastosis virus (Vennstrom and Bishop, 1982). Whereas v-*erb*A alone is not an efficient inducer of transformation of erythroid progenitor cells, v-*erb*A enhances transformation efficiency of other oncogenes (v-*erb*B, v-Ha-*ras*, v-*src* and v-*fms*) by blocking differentiation (Gandrillon *et al.*, 1987). c-*erb*A is an altered form of the normal cellular receptor for thyroid hormone, which acts as a DNA binding transcription factor (Sap *et al.*, 1986; Weinberger *et al.*, 1986). Upon ligand binding the wildtype receptor modulates the expression of target genes to induce cell differentiation. In the presence of ligand, the v-*erb*A oncogene does not modulate gene expression and, in fact, competes with wildtype receptor binding, resulting in a dominant negative effect on cell differentiation (Damm *et al.*, 1989; Sap *et al.*, 1989).

Whereas the anti-oncogenes described above have an intracellular location, a number of secreted factors, including TGF-β, TNF and IFN (see also section on growth factors) have a growth inhibitory action on certain target cells (Wang and Hsu, 1986). The mechanism by which the signals transduced from the cell surface receptors of these growth inhibitors through the cytoplasm to the nucleus are poorly understood.

The identification of tumor suppressor genes (or more general, growth inhibitors) has proven far more difficult than the growth stimulating oncogenes. Typically, an oncogene can score positive in transfection-transformation assays; transfection of extracted tumor DNA into the mouse fibroblast cell line NIH-3T3, can result in the formation of transformed cell foci. Using comparable gene transfer techniques for the cloning of tumor suppressor genes, one has to select for rare, slow growing transfected cells in a background of fast growing transformed ones. Strategies for isolating of tumor (or transformation) suppressor genes (other than Rb-1, WT, p53, c-*erb*A), have been based upon molecular and functional differences between the normal and tumor cell. For example, the cDNA from the Kirsten-*ras*-revertant gene (*Krev*-1) was isolated from a human fibroblast cDNA expression library by virtue of its revertant-inducing activity in the v-Ki-*ras* transformed NIH 3T3 cells (Kitayama *et al.*, 1989). The predicted *Krev*-1 protein is structurally related to *ras* proteins, with GTP-binding domains on the putative effector-interaction domain (Table 3).

c. Interplay between positive and negative growth control

Recently, it was shown that both Rb and p53 anti-oncogene products form stable complexes with the oncogenic proteins of DNA tumor viruses. pRb complexes with SV40 large T antigen, EIA of adenovirus and E7 protein of human papillomavirus (Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989; Egan *et al.*, 1989). p53 binds to E1b of adenovirus, as well as to SV40 T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982). *in vitro* mutation studies with these viral proteins have demonstrated that the interaction domain of pRb with the viral protein is required for transformation (DeCaprio *et al.*, 1988). Interestingly, these domains are also the most frequently sites for naturally occurring mutations found in tumors(Hu *et al.*, 1990). Complexing of the viral proteins with the anti-oncogene products of Rb and p53 may block the normal growth inhibitory actions of these proteins on the untransformed cell.

The phosphorylation state of pRb is cell cycle dependent (DeCaprio *et al.*, 1989; Buchkovich *et al.*, 1989; Chen *et al.*, 1989; Mihara *et al.*, 1989). The under phosphorylated pRb form prevails during the G1 phase of the cell cycle and presumably is the active form, contributing to growth suppression. The potent secreted growth inhibitor TGF-β1 interferes with the cell cycle at late G1 (Heimark *et al.*, 1986; Shipley *et al.*, 1986). Recently, TGF-β1 was shown to block pRb phosphorylation in mink cells (Laiho *et al.*, 1990b). Apparently, therefore, TGF-β and pRb may function via a common growth inhibitory pathway. In keratinocytes, growth inhibition by TGF-β1 appears to involve down-regulation of c-*myc* proto-oncogene expression through inhibition of transcriptional initiation (Pietenpol *et al.*, 1990b). This effect was diminished in keratinocytes transfected with DNA tumor viruses. Viruses with mutant transforming protein that fail to bind pRb (and other cellular proteins) do not show this effect (Pietenpol *et al.*, 1990a). This suggests that pRb, or another protein that interacts with the viral protein binding domain mediates TGF-β induced down-regulation of c-*myc* in keratinocytes.

Both the epidemiology of cancer and genetic analysis of tumors indicate that tumorigenesis is a multi-step process. Mutations in multiple genes either causing the activation of the proto-oncogenes or the inactivation of tumor suppressor genes (or anti-oncogenes) or their combination are necessary to transform a normal cell into a tumor cell (Land *et al.*, 1983). For example, in colorectal cancer, tumorigenesis proceeds through a series of clinical stages, ie. hyperproliferation, three stages of adenomas, carcinoma and metastasis. Each of these stages correlate with concomitant genetic alterations, including *ras* activation and loss of the tumor suppressor genes, p53 (Baker *et al.*, 1989), DCC (an acronym for Deleted in Colorector Carcinoma) and an unidentified gene(s) on chromosome 5 (Fearon *et al.*, 1990; Fearon and Vogelstein, 1990)

VI. Growth Factors in early Embryogenesis

a. Growth factor expression in preimplantation embryos

During embryogenesis, extensive proliferation and differentiation occurs. Growth factors and their signal response elements are likely to play an important role in this process. In early mammalian development, the direct study of growth factors involved in growth and differentiation is difficult, as the embryo consists of only a few thousand cells with multiple cell types, and is rather inaccessible within the maternal uterus. Therefore, a number of model systems have been developed to study growth factors in early embryogenesis.

Preimplantation mouse embryos develop *in vitro* independently of exogenous added growth factors, suggesting that endogenously produced factors are sufficient to support growth (Biggers, 1971). Using a technique termed mRNA phenotyping (based upon the polymerase chain reaction of cDNA with growth factor specific primers) growth factor expression can be detected in individual blastocysts. PDGF-A, TGF-α, IGF-II and TGF-β1 transcripts were found in blastocytes, while EGF, bFGF, NGF and G-CSF were absent (Rappolee *et al.*, 1988). Initially, the amount of PDGF-A and TGF-α transcripts were high, but disappeared at the 2 cell stadium suggesting that they were maternally derived. The expression reappeared once expression in the zygotic genome was initiated. TGF-β1 expression appears only after fertilization. The transcripts were translated as immunofluorescence studies indicated the presence of TGF-β1, PDGF-A and TGF-α proteins. This suggests a role for growth factors in the growth and differentiation of early mammalian embryos.

b. Growth factors regulating embryonic stem cell proliferation and differentiation

Embryonic stem (ES) cells are the totipotent cell lines established from early embryos. When ES cells are injected into a blastocyte they can contribute to all adult tissues, including the germ-cell lineages. In contrast, embryonal carcinomas (EC) cells, derived from spontaneous or experimentally-induced teratocarcinoma tumors, have only a limited differentiation capacity and induce tumors when introduced into embryos (Martin, 1980). Some EC cells are blocked in their differentiation and can be kept in continuous culture. These cells form a useful source for the isolation of growth factors involved in early mammalian development. For example, embryonal carcinoma-derived growth factor (ECDGF), a member of the FGF family, was isolated from the conditioned media of murine PC13EC cells (Heath and Isacke, 1984; Heath *et al.*, 1989).

Differentiation of EC cells is accompanied with changes in growth factor expression. For example, retinoic acid induces differentiation of PC13 into PC13END (resembling extra-embryonic mesoderm) with a concomitant increase in IGF expression, and a cessation of ECDGF expression (Heath and Rees, 1985; Heath and Shi, 1986). During retinoic acid induced differentiation of the EC cell line, Tera-2 clone 13, PDGF-A expression was reduced, but TGF-β1 expression increased (Weima *et al.*, 1988). The expression levels of these two growth factors may possibly be essential for the transformed phenotype and differentiation state of EC cells.

When grown in isolation, ES cells differentiate spontaneously. ES cell differentiation can be prevented by growing these cells on certain mitotically inactivated heterologous feeder layers (Smith and Hooper, 1983; Koopman and Cotton, 1984), such as the Buffalo rat liver (BRL) cells (Smith and Hooper, 1987). An ES differentiation inhibitory activity (DIA) was purified from the conditioned media of the BRL cells, which can substitute for the feeder layer in continuous cultures of ES cells (Smith *et al.*, 1988a). When cells are grown in the absence of DIA, they differentiate into cells with mesodermal characteristics. Treatment with retinoic acid in the presence of DIA results in the differentiation into cells with characteristics of parietal endoderm (Heath *et al.*, 1988). The differentiation pathway appears to be dependent on the combination of regulatory factors and most likely is determined by target cell and microenvironment. *In vivo*, DIA may function as a switch between self-renewal and stem cell differentiation. Structural and functional characterizations of DIA (Smith *et al.*, 1988a) has shown a relatedness (if not identity) of DIA to two other factors described previously; human interleukin for DA cells (HILDA)(Godard *et al.*, 1988) and leukemia inhibitory factor (LIF)(Gearing *et al.*, 1987; Gough *et al.*, 1988). HILDA/LIF has been shown to act by binding to high affinity cell surface receptors on mature hemopoietic cells, as well as ES cells. As with many growth factors, one factor appears to have pleiotropic functions, retarding the differentiation of ES cells (Williams *et al.*, 1988; Smith *et al.*, 1988a), supporting the growth of a murine IL-3 sensitive cell line (DA_2) and inducing the differentiation of M1 cells (Hilton *et al.*, 1988).

c. Mesoderm induction by growth factors

In the early embryo, mesoderm differentiates from ectoderm upon inductive interactions from the underlying endoderm. Most mechanistic studies have been performed with amphibian embryos, which are technically easier to manipulate than the inaccessible intra-uterine mammalian embryos. Ectoderm alone, dissected from the blastula of *Xenopus* embryos, forms a mass of epidermal cells. However, when dissected ectoderm is combined with dissected endoderm, mesoderm is formed (Nieuwkoop, 1969). The endoderm derived signals appear to be diffusible and isolated growth factors have been shown to mimic the mesoderm inducing signals. When isolated *Xenopus* animal cap explants are treated with FGF family members

(aFGF, bFGF, ECDGF, KGF and *int*-2 protein product)(Slack *et al.*, 1987; Paterno *et al.*, 1989) and mammalian TGF-β family members (activin A, TGF-β2 and TGF-β3), mesoderm is formed (Smith *et al.*, 1988c; Rosa *et al.*, 1988; Smith *et al.*, 1990; van den Eijden-van Raaij *et al.*, 1990; ten Dijke, unpublished results). Different members of each family exhibit different potencies. Synergism is found between TGF-β and the FGF family of growth factors (Kimelman and Kirschner, 1987). Furthermore, the type of mesoderm that is induced appears to be dependent on concentration. The role of these factors in the natural inductive process has not yet been directly demonstrated. However, genes homologous to both mammalian TGF-β (Weeks and Melton, 1987; Kondaiah *et al.*, 1990) and FGF (Kimelman and Kirschner, 1987) have been identified from frog libraries, and are expressed in embryonal stages when mesoderm induction takes place. The *Xenopus* activin, TGF-β2 and TGF-β5 have been isolated from *Xenopus* XTC conditioned medium (Smith *et al.*, 1988c; Roberts *et al.*, 1990). FGF has been isolated from *Xenopus* blastula in an amount that would be sufficient for mesoderm induction *in vivo* (Kimelman *et al.*, 1988). The demonstration that mammalian growth factors can mimic amphibian mesoderm induction signals indicates a considerable evolutionary conservation of growth factors and suggest a role for those factors in early mammalian development.

VII. Concluding Remarks

Within recent years, substantial progress has been made in our understanding of the molecular mechanisms of cellular growth during tissue homeostasis and development. There appears to be a direct relationship between these processes as similar growth factor and receptor/signaling pathways are being utilized. However, many steps in the inter- and intracellular signaling pathways remain virtually unknown.

As different pathways in the growth control process are elucidated, new insights will be obtained not only in the development of complex organisms from fertilized eggs, but also in the origins of uncontrolled growth. Understanding the pivotal role of growth factors in these processes will contribute to an improved diagnosis and prognosis, and will lead to the development of new strategies for therapeutic intervention and prevention of neoplasia.

VIII. References

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CHAPTER THREE

Transforming Growth Factor-βs: Polypeptides with Multiple Biological Functions

TRANSFORMING GROWTH FACTOR-βS: POLYPEPTIDES WITH MULTIPLE BIOLOGICAL FUNCTIONS

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I. Introduction

Transforming growth factor (TGF) was a term originally used to describe an activity produced by murine sarcoma virus-transformed cells (De Larco and Todaro, 1978). Medium conditioned by these cells was able to confer morphological transformation and anchorage independence on certain target cells, which are characteristics that often correlate with tumorigenesis of cells *in vivo* (Cifone and Fidler, 1988). TGF induced transformation was shown to be reversible, as "transformed" cells reverted back to normal upon removal of TGF. Subsequently, similar transforming activities were also found in the conditioned media of other tumor cells (Todaro *et al.*, 1980) as well as in normal tissues, particularly when epidermal growth factor (EGF) was added to the assay (Roberts *et al.*, 1981). Purification of this activity revealed that it was not a single biochemical entity, but was derived from two distinct proteins, termed TGF-α and TGF-β. Both proteins alone were unable to induce soft agar growth of normal rat kidney NRK fibroblast, but act together synergistically (Anzano *et al.*, 1983).

TGF-α and TGF-β are different molecules in structure and function. TGF-α is structurally related to EGF and competes with EGF receptor binding (Todaro *et al.*, 1980). TGF-β is a multifunctional dimeric protein of 25 kDa (for recent reviews see Roberts and Sporn, 1990; Lyons and Moses, 1990) and its structure, functions and mechanism of action are the topic of this review. TGF-β may act to stimulate or inhibit cell proliferation and differentiation or regulate other functional processes. Multiple forms of TGF-β have been identified which share similar, but not identical biological activities. Five true different

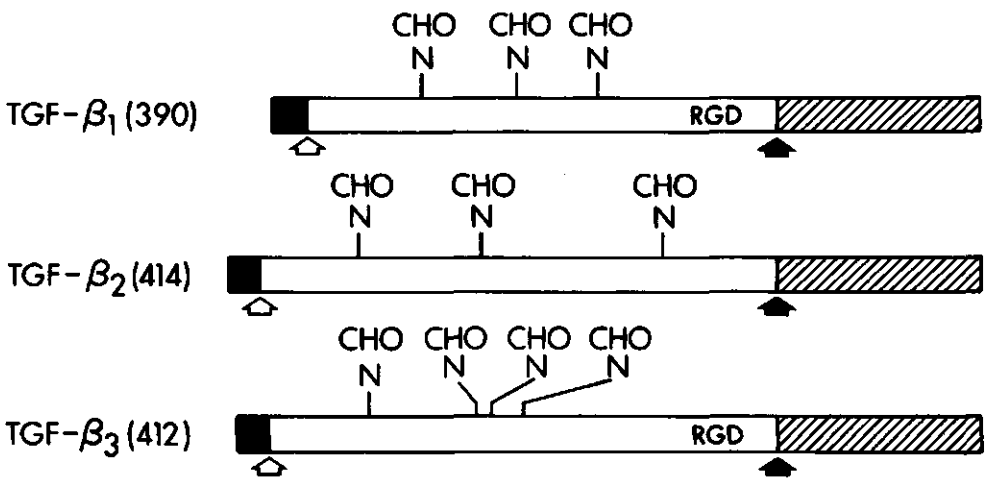

Figure 1

Schematic representation of the human TGF-β1 (Derynck *et al.*, 1985), TGF-β2 (de Martin *et al.*,1987) and TGF-β3 (ten Dijke *et al.*, 1988; Derynck *et al.*, 1988). The relative positions of the N-glycosylation sites (N-CHO) and the potential fibronectin cellular recognition site (RGD) are indicated. The arrow heads indicate the cleavage sites following the signal peptide and preceding the C-terminal mature TGF-β3 sequences (shaded). The size of the precursor protein in amino acids is indicated between brackets.

TGF-β genes have been described in vertebrates. Moreover, TGF-βs are part of a larger super family of structurally related, but functionally different proteins. TGF-βs are produced by many different cell types and most cell types have high affinity specific receptors, suggesting a broad physiological importance. The clinical implications of the role of TGF-βs in disease and therapy are also discussed.

II. Molecular Biology and Biochemistry of TGF-βs

Identification and Structure of TGF-β1

TGF-β1 was first purified to homogeneity from human platelets (Assoian *et al.*, 1983), human placenta (Frolik *et al.*, 1983) and bovine kidney (Roberts *et al.*, 1983), using the normal rat kidney (NRK) fibroblast soft agar assay for detection. TGF-β1 was identified as a disulfide linked dimer of 25 kDa. Upon reduction two identical subunits of 12 kDa were obtained with no biological activity. The cloning and DNA sequencing of the human TGF-β1 cDNA revealed that TGF-β1 is synthesized as a larger pre-pro-precursor protein (Derynck *et al.*, 1985) that undergoes at least two proteolytic cleavage steps for maturation in a biologically active protein. The pre-pro-TGF-β1 contains a hydrophobic signal peptide of 29 amino acids at the N-terminus. The pro-TGF-β1 is further proteolytically processed at a tetrabasic RHRR cleavage site yielding the 112 amino acid C-terminal mature fragment.

After secretion and processing the 25 kDa TGF-β1 mature protein remains non-covalently associated with the N-terminal precursor fragment (Gentry *et al.*, 1987). *In vitro*, both chaotropic agents and specific proteases can disrupt these interactions, releasing biologically active TGF-β1 (Lawrence *et al.*, 1985; Lyons *et al.*, 1988). Plasmin was shown to cleave the N-terminal precursor fragment, which may result in a conformational change that destabilizes the latent complex (Lyons *et al.*, 1990). The N-terminal precursor fragment contains several N-linked glycosylation sites (Brunner *et al.*, 1988). Recently, it was shown that alterations in glycosylation can also lead to activation of latent TGF-β1 (Miyazono and Heldin, 1989).

TGF-β-like factors

TGF-β-like factors have been isolated many times, by virtue of its multifunctional character and the existence of multiple forms. In bovine bone two distinct forms were identified, using an assay that measures the induction of cartilage extracellular matrix proteins (Seyedin *et al.*, 1985). Upon sequence determination cartilage-inducing factor A and B (CIF-A and CIF-B) were shown to be identical to TGF-β1 and TGF-β2, respectively (Seyedin *et al.*, 1986; Seyedin *et al.*, 1987). Subsequently, TGF-β2 was isolated from porcine platelets (Cheifetz *et al.*, 1987) and from several conditioned media. A potent T-cell suppressor factor secreted by glioblastoma cells (de Martin *et al.* 1987), a growth inhibitor secreted by monkey BSC-1 kidney cells (Hanks *et al.*, 1988) and a TGF-β-like factor from a prostatic adenocarcinoma cell line (Ikeda *et al.*, 1987) were all shown to be identical to TGF-β2.

The cDNAs for three different TGF-βs, termed TGF-β3 (ten Dijke *et al.*, 1988a; Derynck *et al.*, 1988; Jakowlew *et al.*, 1988a), TGF-β4 (Jakowlew *et al.*, 1988b) and TGF-β5 (Kondaiah *et al.*, 1990) have recently been isolated. Whereas TGF-β3, like TGF-β1 and TGF-β2 are found in mammals (see also figure 1), TGF-β4 and TGF-β5 have thus far only been identified in chicken and frog, respectively (Jakowlew *et al.*, 1988b; Kondaiah *et al.*, 1990). The TGF-β3 protein was recently expressed in CHO cells transfected with TGF-β3 cDNA expression plasmids (Graycar *et al.*, 1989; ten Dijke *et al.*, 1990b) and the TGF-β5 protein was isolated from medium conditioned by *Xenopus* cultured cells (XTC)

(Roberts *et al.*, 1990). The TGF-β4 putative protein has not yet been isolated. Heterodimers may also be formed, as TGF-β1.2, containing one TGF-β1 and one TGF-β2 chain was identified in porcine platelets (Cheifetz *et al.*, 1987). This increases even more the number of possible forms of TGF-β, as multiple TGF-βs are often expressed in one cell.

As predicted from their cDNA sequence, each of the TGF-βs are produced as larger precursor proteins with N-linked glycosylation sites in the precursor segments. All TGF-βs have a signal peptide sequence at the N-terminus, except TGF-β4, which may function within the cell. Proteolytic processing at the tetra basic cleavage site yields for each TGF-β a mature protein of 112 amino acids, except TGF-β4 which has a mature protein of 114 amino acids. The mature forms share between 70-80% sequence identity with a strict conservation of all nine cysteines. In most assays, TGF-βs have similar biological activities, including growth inhibition of MV1Lu mink lung epithelial cells and induction of NRK soft agar growth (Roberts *et al.*, 1990; Graycar *et al.*, 1989; ten Dijke, *et al.*, 1990b; Cheifetz *et al.*, 1990).

Transcriptional regulation

Northern blot analysis and *in situ* hybridization studies reveal distinct temporal and spatial expression patterns for each TGF-β, suggesting that each isoform is differently transcriptionally regulated. In the TGF-β1 transcription unit two major transcriptional start sites are present with multiple positive and negative regulatory elements. Two Sp1 and three AP-1 binding sites have been identified in the human TGF-β1 promoter regions (Kim *et al.*, 1989). The ability of TGF-β1 (and also c-*jun*) to upregulate TGF-β1 expression was found to be mediated by binding of AP-1 (Jun-Fos) complex in both promoter regions. The induction of c-*jun* by TGF-β and auto induction of c-*jun* may play a role to amplify and sustain the action of TGF-β in normal physiological and pathological processes (Kim *et al.*, 1990). The receptors for steroids and retinoids and may also play an important role in transcriptional regulation of TGF-βs, as suggested by induction of TGF-β2 by retinoic acid in primary cultures of mouse keratinocytes (Glick *et al.*, 1990) and estrogen induced reduction of TGF-β2 and TGF-β3 expression in estrogen receptor positive (ER+) breast cancer cells (Arrick *et al.*, 1990).

Evolutionary conservation

TGF-β1 is very highly conserved in evolution. The C-terminal 112 amino acids of TGF-β1 are identical in human (Derynck *et al.*, 1985), porcine (Derynck and Rhee, 1989), bovine (Van Obberghen - Schilling *et al.*, 1987), chicken (Jakowlew *et al.*, 1988c) and simian species (Sharples *et al.*, 1987) and differ by only one amino acid in the murine form (Derynck *et al.*, 1986). The same high degree of conservation is found for the mature forms of TGF-β2 (de Martin *et al.*, 1987; Madisen *et al.*, 1988; Hanks *et al.*, 1988; Cheifetz *et al.*, 1987; Seyedin *et al.*, 1985; Miller *et al.*, 1989b)) and TGF-β3 (ten Dijke *et al.*, 1988a, ten Dijke *et al.*, 1988b; Derynck *et al.*, 1988; Jakowlew *et al.*, 1988a; Miller *et al.*, 1989a). The N-terminal domains for each TGF-β are also highly conserved (~80-90% amino acid sequence identity), suggesting that this part of the protein may have an important function, including proper folding, disulfide bond formation and in the export the TGF-β mature protein (Gray and Mason, 1990).

Comparing the gene structure of TGF-β1 and TGF-β3 reveals that all intron-exon junctions are at the same positions, with the exception of the first intron, which differs by three nucleotides. This suggests that the TGF-βs originate from the same ancestral gene (Derynck *et al.*, 1987; Derynck *et al.*, 1988). The human genes fo TGF-β1, TGF-β2 and TGF-β3 have been localized on different chromosomes (Fujii *et al.*, 1986; Barton *et al.*,

TABLE 1: The TGF-β Superfamily[1]

Growth Factor	Abbreviation	Function
Transforming Growth Factor-β1	TGF-β1	Multifunctional regulators of cell growth, differentiation and function
Transforming Growth Factor-β2	TGF-β2	
Transforming Growth Factor-β3	TGF-β3	
Transforming Growth Factor-β4 (chicken)	TGF-β4	
Transforming Growth Factor-β5 (frog)	TGF-β5	
Activin A (βAβA) or XTC-mesoderm *inducing factor (frog)*	XTC-MIF	- Stimulators of FSH secretion by pituitary cells; inducers of mesoderm during frog development; inducers of erythroid differentiation;
Activin AB (βAβB)		
Activin B (βBβB)		
Inhibin A ($\alpha\beta$A)		- Inhibitors of FSH secretrion by pituitary cells
Inhibin B ($\alpha\beta$B)		
Mullerian inhibiting substance	MIS	Induces regression of the Mullerian ducts in male embryos
Decapentaplegic gene complex (*Drosophila*)	*DPP-C*	Involved in pattern formation in *Drosophila*
Vegetal protein-1 (frog)	*Vg*-1	Involved in mesoderm induction
Bone morphogenetic protein 2	BMP-2	Involved in cartilage and bone formation
Bone morphogenetic protein 3 or osteogenin	BMP-3	
Bone morphogenetic protein 4	BMP-4	
Bone morphogenetic protein 5	BMP-5	
Bone morphogenetic protein 6 or *Vg*-1 related gene	BMP-6 *Vgr*-1	
Bone morphogenetic protein 7 or osteogenic protein-1	BMP-1 OP-1	

[1] All listed growth factors have been identified in mammals, unless stated otherwise.

1988; ten Dijke *et al.*, 1988b), suggesting that after amplification, these genes have dispersed and subsequently diverged in sequence.

TGF-β superfamily

TGF-βs form part of a larger superfamily of proteins that structurally as well as functionally are distantly related (see Table 1). The two forms of inhibin and the three forms of activin, which were first identified as gonadal proteins that regulate pituitary secretion of follicle stimulating hormone (Ling *et al.*, 1986; Mason *et al.*, 1985) and the Mullerian inhibiting substance of MIS, which causes regression of the female rudiments in the developing male reproductive system (Cate *et al.*, 1986) share approximately 60% identity with the TGF-β mature forms. An even more distantly related group, with approximately 30-40% identity with TGF-β mature forms include the *decapentaplegic* gene complex (*DPP-C*) involved in dorsal-ventral pattern formation in the developing *Drosophila* embryo (Padgett *et al.*, 1987), the *Vg*-1 gene product expressed in frog oocytes and possibly involved in mesoderm formation (Weeks and Melton, 1987), the mouse *Vg-1R* gene product (Lyons *et al.*, 1989) and bone morphogenetic proteins (BMP's)(Wodzney *et al.*, 1988) which play a role in cartilage and bone formation. All members are produced as larger precursor proteins with their mature biologically active parts at the C-terminus and with seven of the nine cysteine residues conserved among all members.

III. TGF-β receptors and binding proteins

TGF-β receptors

The action of TGF-βs, like all growth factors, is mediated through binding to specific cell surface receptors. Virtually all cell types examined to date carry TGF-β receptors, with the exception of retinoblastoma and pheochromocytoma cells, and bind TGF-βs with affinities in the pico molar concentration range (Massague and Like, 1985; Wakefield *et al.*, 1987; Kimchi *et al.*, 1988). By covalently linking radiolabeled TGF-β to cell surface molecules followed by polyacrylamide gel electrophoresis of the affinity labeled complexes three distinct classes of cell surface proteins (in most cases) are identified, named receptor type I and II and a proteoglycan termed betaglycan. The relative distribution of the three cell surface proteins types varies per cell type (Massague *et al.*, 1990).

The TGF-β receptor types I and II are glycoproteins which appear as 65 kDa and 85-110 kDa affinity labeled complexes on reduced polyacrylamide gels (Massague, 1985; Cheifetz *et al.*, 1986) and have a high affinity for TGF-β1 (Kd=5-50 pM) and are present in low numbers (300-400 binding sites) per cell. The betaglycan is a heterogenous proteoglycan with a molecular weight of 280-300 kDa of affinity labeled complex (Massague, 1985) and consists of 200 kDa of glycosaminoglycan (GAG) chain mass and 10 kDa of N-linked glycans attached to a heterogeneous core component of 100-120 kDa (Cheifetz *et al.*, 1988a; Segarini and Seyedin, 1988). The GAG's are not required for functional expression of the receptor on the cell surface (Cheifetz and Massague, 1989). The affinity of TGF-β1 for the proteoglycan is lower than for the glycoproteins (Kd=30 ~ 300 pM) with up to 10^5 binding sites per cell (Cheifetz *et al.*, 1987; Cheifetz *et al.*, 1988a). The biological effects of TGF-βs appear to be mediated by type I and/or type II receptors (Boyd and Massague, 1989; Laiho *et al.*, 1990b) (see also mechanism of action). None of the receptor types show affinity for latent TGF-β. A fourth receptor type has, thus far, only been detected in a pituitary tumor cell line and has a unique property among TGF-β receptor types in that it binds inhibin and activin (Cheifetz *et al.*, 1988b).

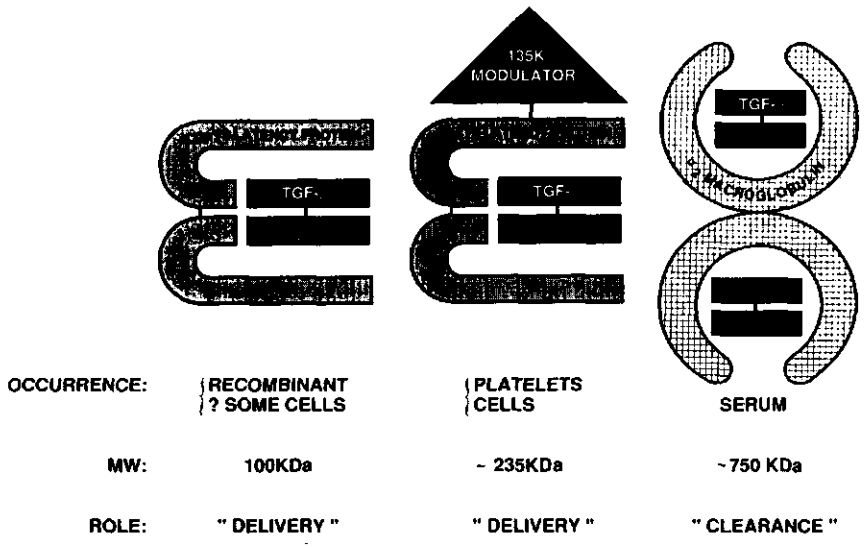

Figure 2

Latent forms of TGF-β1. Latent TGF-β1 secreted by recombinant cells is a non-covalent complex of the 75 kDa dimeric latency associated protein (LAP) derived from the TGF-β1 precursor and the processed dimeric 25 kDa TGF-β1 (small latent complex). As secreted from cells and released from platelets, the large latent complex contains in addition a 135 kDa modulator protein, termed latent TGF-β binding protein (LTBP). The complex of α2-macroglobulin with the mature dimeric TGF-β is also latent and probably functions as a clearance mechanism. (from Wakefield *et al.*, 1989, reprinted with permision of the authors).

TGF-β binding proteins

TGF-βs are synthesized as latent complexes with high molecular weights (Pircher *et al.*, 1986)(figure 2). The TGF-β1 latent complex isolated from platelets consists of the mature TGF-β1 (25 kDa) non-covalently associated with a disulfide-bonded complex of a dimer of the N-terminal propeptide of the TGF-β1 precursor (75 kDa) (TGF-β1-latency associated peptide) and a third component termed latent TGF-β1 binding protein (LTBP)(135 kDa)(Miyazono *et al.*, 1988; Wakefield *et al.*, 1988). *In vitro* TGF-β1 can be released in a biologically active form from the latent complex by treatments with extreme pH values, chaotropic agents and proteases such as plasmin and cathepsin D and by enzymatic removal of the carbohydrate structures of TGF-β1 precursor (Miyazono *et al.*, 1990; Lawrence *et al.*, 1985: Lyons *et al.*, 1988). Recombinant TGF-β1 expressed in CHO cells, is also produced in a latent form (Gentry *et al.*, 1987; Wakefield *et al.*, 1989). However, the LTBP was not associated with the latent complex (figure 2). Apparently, the N-terminal precursor region is sufficient to confer latency on the active TGF-β1 and has been shown to function as a functional binding protein to regulate biological activity of TGF-β1 (Gentry and Nash, 1990). The latent complex which includes LTBP is termed the large latent complex, whereas the complex without LTBP is termed small latent complex (Wakefield *et al.*, 1989). Recently, the LTBP cDNA was cloned from a fibroblast library (Kanzaki *et al.*, 1990). Sequence analysis revealed the existence of 16 EGF-like repeats and three copies of an unknown motif. The LTBP did not bind or inactivate TGF-β1, also indicating that the binding protein is not necessary for TGF-β1 latency. Possibly the binding protein could play a role in stabilization, targeting and activation of the latent complex (Kanzaki *et al.*, 1990).

In serum, another latent complex can be isolated, consisting of TGF-β and α2 macroglobulin (O'Connor-McCourt and Wakefield, 1987; Wakefield, *et al.*, 1988, Huang *et al.*, 1988). This complex is different from the cell secreted latent TGF-β complex in that it contains only processed mature TGF-β (figure 2). The latent platelet TGF-β1 does not bind to α2 macroglobulin. Possibly, α2 macroglobulin may function as a scavenger for excess of TGF-βs, in order to restrict the action range of TGF-βs *in vivo*.

TGF-βs can also interact with a soluble form of the betaglycan, that lacks the membrane anchor (Andres *et al.*, 1989). This form is secreted by some cells and found in low amounts in serum and in extracellular matrices. It is not known whether binding of TGF-βs to betaglycan inhibits the activity of the growth factor. Possibly, betaglycan may act as a storage or clearance system for biologically active TGF-βs and play a role in cell to cell recognition and adhesion (Massague *et al.*, 1990). Recently, decorin, a small proteoglycan, consisting of a core protein and a single glycosaminoglycan chain, was shown to bind TGF-β1 and to neutralize its activity (Yamaguchi *et al.*, 1990). TGF-β1 was shown to stimulate the synthesis of decorin, suggesting that it may function as a negative feedback mechanism.

IV. Effects of TGF-βs

Effects on proliferation

TGF-βs can both stimulate and inhibit cell proliferation. Whereas most mesenchymal derived cells are growth stimulated (Kimura *et al.*, 1989; Hill *et al.*, 1986; Centrella *et al.*, 1987, Vivien *et al.*, 1990; Battegay *et al.*, 1990), epithelial (Coffey *et al.*, 1988a), endothelial (Baird and Durkin, 1986) and lymphoid (Kehrl *et al.*, 1986a,b) cells are mostly inhibited by TGF-βs. However, both growth stimulation and inhibition by TGF-βs have been observed for the same cell type *in vitro*. The particular effect of TGF-βs appears to be dependent on not only cell type, but also cell culture conditions and

presence of other factors (Roberts and Sporn, 1990). Most of the published work on the effects of TGF-βs have been performed with TGF-β1 and as all TGF-βs associate with the receptor types I and II and betaglycan, the results on TGF-β1 will most likely, at least qualitatively, apply for the other TGF-βs (unless stated otherwise).

TGF-β1 was initially discovered as a growth stimulator of NRK in soft agar, in the presence of TGF-α (De Larco and Todaro, 1978). Human marrow fibroblasts and early passage fetal fibroblasts (Kimura *et al.*, 1989; Hill *et al.*, 1986) and other connective tissue cells, such as osteoblasts (Centrella *et al.*, 1987), chondrocytes (Vivien *et al.*, 1990) and smooth muscle cells (Battegay *et al.*, 1990) are also growth stimulated by TGF-βs, with low concentration being more mitogenic than high concentrations.

In vivo TGF-βs also stimulates mesenchymal cell growth. Subcutaneous injection of TGF-β1 in neonatal mice stimulates the formation of granulation tissue consisting of a dense population of fibroblasts, macrophages and neovasculature (Roberts *et al.*, 1986). TGF-β1 injected into rat femurs resulted in intramembranous bone formation and chondrogenesis (Joyce *et al.*, 1990b). These results suggests a physiological role of TGF-β in wound healing and bone formation. Indeed, many cells involved in tissue repair, including activated marcrophages, lymphocytes and fibroblasts, express TGF-βs and the highest levels of TGF-βs in the body are found in platelets and bone (Roberts and Sporn, 1990). Moreover, in a closed space/wound chamber model, the endogenous level of TGF-β peaks during the phase of wound healing associated with maximal fibroblast and collagen synthesis (Cromack *et al.*, 1987; Grotendorst *et al.*, 1988). Expression of endogenous TGF-β is induced in a bilateral femur fractures in the rat and correlated with areas of mesenchymal cell proliferation and differentiation into both osteoblasts and chondrocytes (Joyce *et al.*, 1990a,b). TGF-β like activities are released after bone resorption and may regulate bone formation and resorption during remodeling. The acidic environment surrounding the osteoclast may provide a mechanism of activation of the secreted latent TGF-βs (Oreffo *et al.*, 1989).

The growth inhibitory action of TGF-βs on many cells *in vitro* appears to function by its ability to antagonize the effects of other growth factors. For example, TGF-β1 inhibits the proliferation of EGF stimulated keratinocytes (Coffey *et al.*, 1988a), inhibits the mitogenic effect of FGF on vascular endothelial cells (Baird and Durkin, 1986), inhibits the mitogenic effects of IL-2 on T and B lymphocytes (Kehrl *et al.*, 1986a, b), inhibits the mitogenic effect of PDGF on rat embryo fibroblasts (Anzano *et al.*, 1986).

In vivo TGF-βs can also act as a growth inhibitor. For example, TGF-β1 in a slow release carrier, implanted in the mammary gland of mice caused the growth inhibition of epithelial ductal end buds (Silberstein and Daniel, 1987). Intravenously administered TGF-β1 and TGF-β2 inhibited the liver regeneration following partial hepatectomy, suggesting a physiological role of TGF-βs in the quiescence of normal hepatocytes (Russel *et al.*, 1988).

Effects of TGF-βs *in vitro* do not necessarily correspond with the *in vivo* effects. An example is the effect of TGF-βs on endothelial cells. *In vitro* TGF-β1 inhibits endothelial cell growth in monolayer (Frater-Schroder *et al.*, 1986). *In vivo* TGF-β1 stimulates the formation of new blood vessels (angiogenesis), possibly in part, by attraction and activation of macrophages, which release angiogenic factors (Roberts *et al.*, 1986).

Other effects of TGF-βs

TGF-βs affect the differentiation of many cell types *in vitro*. TGF-β1 is a potent regulator of myogenesis inhibiting the fusion into multinucleated myotubes and the

expression of muscle specific genes in myoblasts (Massague *et al.*, 1986; Olson *et al.*, 1986). TGF-β1 also inhibits the differentiation of adipocytes (Ignotz and Massague, 1986), inhibits immunoglobulin secretion by B lymphocytes (Kehrl *et al.*, 1986) and inhibits steroid production in Leydig cells and adrenocortical cells (Avallet *et al.*, 1987; Feige *et al.*, 1987) However, TGF-β1 stimulates the differentiation of bronchial epithelial cells (Masui *et al.*, 1986), keratinocytes (Reiss and Satorelli, 1987) and intestinal epithelial cells (Kurokawa *et al.*, 1987).

Extracellular matrix (ECM) is produced by many cell types and in almost all cases TGF-βs stimulate the EM formation, either by increasing the production and/or decreasing the degradation of the matrix. The transcription of multiple genes involved in ECM turnover is affected by TGF-βs. TGF-β1 increases the transcription of genes encoding for matrix components such as type I and type III collagen, fibronectin (Ignotz and Massague, 1986; Ignotz *et al.*, 1987) and chondroitin (Bassols and Massague, 1988) in several cell types, osteonectin (Noda and Rodan, 1987), osteocalcin (Noda, 1989) and osteopontin (Noda *et al.*, 1988) in osteosarcomas and thrombospondin (Wrana *et al.*, 1986) and tenascin (Pearson *et al.*, 1988) in fibroblast cells and matrix receptors such as integrins (Ignotz and Massague, 1987) in several cell types. Several growth factors including FGF and EGF induce the transcription of genes encoding metalloproteases like collagenase and stromelysin in fibroblasts. TGF-β1 blocks the transcriptional induction of these genes (Kerr *et al.*, 1988; Edwards *et al.*, 1987). Moreover, TGF-β1 in combination with EGF stimulates tissue inhibitor of metalloproteinases TIMP expression (Edwards *et al.*, 1987) and TGF-β1 by itself can induce the synthesis of plasminogen activator inhibitor I (PAI-1)(Lund *et al.*, 1987).

TGF-β1 is a potent chemoattractant for monocytes and fibroblasts (Wahl *et al.*, 1987; Postlethwaite *et al.*, 1987) with an effective concentration below 10 pg/ml. This dose is much lower than what is required for regulation of proliferation and differentiation and extracellular matrix production. High concentrations of TGF-β1 are not chemotactic. The effect is also observed *in vivo* as a local injection of TGF-β1 in several different tissue types results in chemotaxis (Roberts *et al.*, 1986; Wiseman *et al.*, 1988; Mustoe *et al.*, 1987).

TGF-βs have immunosuppressive and anti-inflammatory effects; TGF-β1 is growth inhibitory for T and B cells (Kehrl *et al.*, 1986a,b), inhibits the formation of lymphokine activated killer (LAK) cells (Kupper *et al.*, 1988) and the generation of cytolytic T cells (CTC) (Jin *et al.*, 1989). Furthermore, TGF-β1 deactivates H_2O_2 and O_2^- production (Tsunawaki *et al.* ,1988).

Differential effects of TGF-βs

Marked *in vitro* differences in biological potency of TGF-βs have been observed in certain cell types. TGF-β1 has been reported to be approximately 100 times more effective than TGF-β2 in the inhibition of colony formation by hematopoietic progenitor cells (Ohta *et al.*, 1987) and endothelial cells are strongly growth inhibited by TGF-β1 and TGF-β3, but not TGF-β2 (Cheifetz *et al.*, 1990). Furthermore, TGF-β2 and TGF-β3 induce *Xenopus* mesoderm formation, while TGF-β1 alone does not show an effect (Rosa *et al.*, 1988; ten Dijke *et al.*, unpublished results). The *in vitro* differences in potency may be due to differences in recognition by receptors (Cheifetz *et al.*, 1990) and in the susceptibility to antagonism or inactivation by extracellular factors (Danielpour and Sporn, 1990). The *in vitro* differences between TGF-βs are likely to result in different *in vivo* actions.

V. Mechanism of Action

TGF-βs have multifunctional properties affecting many cell types. Several mechanisms may contribute to the cell specific responses to this factor. The biphasic growth stimulatory effect of TGF-βs on chondrocytes, smooth muscle cells and osteoblasts appears to be mediated by a complex control of an autocrine PDGF-A loop. TGF-β1 stimulates PDGF-A mRNA and the secretion of PDGF protein in AKR-2B fibroblasts (Leof *et al.*, 1986). This together with the delayed kinetics of TGF-β1 growth stimulation compared with the direct affect after addition of PDGF suggest that TGF-β1 acts by a PDGF mediated autocrine growth stimulatory loop. Moreover, Battegay *et al.*, recently demonstrated that in smooth muscle cells neutralizing PDGF-A antibodies inhibit the induced proliferation by TGF-βs. They also showed that high doses of TGF-βs decrease the expression of PDGF-receptor α subunits, which are required for biological activity of PDGF-AA. This effect reduces the proliferative response of TGF-βs and correlates with high doses of TGF-βs being less mitogenic (Battegay *et al.*, 1990).

The action of TGF-βs in other systems may also be, in part, due to receptor modulation. TGF-β1 decreases the number of high affinity EGF receptor in endothelial cells (Takehara *et al.*, 1987), and decreases the number of high affinity receptors for EGF and FGF in EGF and FGF stimulated osteosarcoma cells (Mioh and Chen, 1989). Furthermore, TGF-β1 inhibits IL-2 induced upregulation of IL-2 and transferrin receptors in IL-2 induced stimulation of T lymphocytes (Kehrl *et al.*, 1986b), which correlates with the inhibitory effect of TGF-β on T-cell clonal expansion. TGF-β1 downregulates the expression of angiotensin II receptors on adrenocortical cells (Feige *et al.*, 1987) and of hCG receptors on Leydig cells (Avallet *et al.*, 1987), which correlates with inhibitory effects on steroidogenesis in these cells.

However, TGF-β1 does not inhibit EGF receptor binding or any of the early growth factor induced events in EGF-stimulated keratinocytes (Coffey *et al.*, 1988b). Apparently, TGF-β1 interferes with EGF stimulated proliferation at sites more distal than the receptor level. TGF-β1 rapidly reduces c-*myc* expression in keratinocytes (Coffey *et al.*, 1988b). c-*myc* expression appears to be essential for keratinocyte proliferation, as antisense c-*myc* oligonucleotides inhibit keratinocyte growth with the same kinetics as TGF-β1 (Pietenpol *et al.*, 1990b).

In some cases, the effects of TGF-βs appear also mediated, by the stimulatory effect of TGF-βs on the synthesis of extracellular matrix proteins, such as fibronectin. For example, exogenously added fibronectin inhibits the growth of endothelial cells (Madri *et al.*, 1988) and inhibits the differentiation of myoblasts, chondrocytes and pre-adipocytes (Podleski *et al.*, 1979; Rosen *et al.*, 1988; Spiegelman and Ginty, 1983). TGF-β1 has similar effects on these cell types.

TGF-βs mediate their action by binding to receptors. In most cells, three TGF-β binding cell surface proteins are identified termed types I and II and betaglycan (see also TGF-β receptors and binding proteins). Current evidence suggests type I and II receptors to be involved in receptor signal-transduction. The type I and II receptors are detected on all cells that respond to TGF-β, whereas the betaglycan is not detectable in many epithelial, endothelial, lymphoid-derived and monocytic cells that respond to TGF-βs (Segarini *et al.*, 1989). Furthermore, some mutagenized mink cells selected for resistance to growth inhibition by TGF-β show no or anomalous binding to type I or type I and II receptors. The betaglycan is not affected (Boyd and Massague, 1989; Laiho *et al.*, 1990b). In receptor competition studies using ^{125}I-TGF-β1 tracer amounts, the binding to receptor types I and II can be much more efficiently competed by TGF-β1 (and TGF-β3) than TGF-β2 in all cell lines tested. Whereas this differential effect correlates with the activity of TGF-βs on hemapoietic progenitor and vascular endothelial cells, it is

inconsistent for many other effects of TGF-β on cells which correspond similarly (with less than 5 fold difference in potency) to all three factors (including growth regulation by TGF-β of mink cells and osteoblasts). Recently, Cheifetz *et al.* identified a subset of receptor types I and II on mink cells, which has a high affinity for TGF-β2. This receptor subset could not be detected on vascular endothelial cells. This suggests that the cell specific differences in high affinity TGF-β2 receptors may lead to the cell specific differences in responsiveness to this isoform (Cheifetz *et al.*, 1990).

The intracellular pathways of signal transduction after TGF-β receptor binding through the cytoplasm to the nucleus and the effects on gene transcription are virtually unknown. Thus far no consistent involvement of TGF-βs with any of the known signal transduction pathways has been established. Effects by TGF-β1, appear to occur without activation of tyrosine protein kinases (Fanger *et al.*, 1986), S6 protein kinase (Like and Massague, 1986), protein kinase C (Daniel and Fen, 1988), phosphoinositol breakdown and activation of Na^+/H^+ antiporter (Chambard and Pouyssegur, 1987). The intracellular cAMP levels are not affected by TGF-β1 (Kerr *et al.*, 1988), and although TGF-β1 can activate phosphatidyl inositol turnover and induce Ca^{2+} fluxes in Rat-1 cells (Muldoon *et al.*, 1988) these are probably secondary responses. Recent studies suggest the involvement of GTP-binding proteins in the proliferative responses of TGF-β1 in mouse AKR-2B fibroblasts (Murthy *et al.*, 1988). Pertussis toxin inhibits growth stimulation by TGF-β1 and TGF-β1 stimulates GTP-ase activity. Moreover, this study also showed that in the presence of Gpp(NH) the non hydrolysable analog of GTP, the receptor binding affinity of TGF-β1 is reduced, providing strong evidence for a functional association between TGF-β receptor and a G protein.

Ultimately, the result of TGF-β1 on target cells is the change in gene expression. The transcription of many genes is affected, including nuclear proto-oncogenes (c-*myc*, c-*fos*, c-*jun* and *jun*B) (Pertovaara *et al.*, 1989) genes encoding for growth factors (TGF-β, PDGFA and PDGF-B)(Van Obberghen-Schilling *et al.*, 1988; Bascom *et al.*, 1989; Starksen *et al.*, 1987; Leof *et al.*, 1986) and growth factor receptors (EGF and PDGF receptors)(Fernandez-Pol *et al.*, 1987; Gronvald *et al.*, 1989), genes encoding for components of the immune response (IgE receptor, interferon γ and IgK light chain)(Tanaka *et al.*, 1989; Esperik *et al.*, 1987; Briskin *et al.*, 1988) and genes encoding for proteins involved in extracellular matrix formation (see effects of TGF-β). Induction of gene expression can be within minutes of exposure, as is found with c-*fos* (Pertovaara *et al.*, 1989) or can start after a lag phase of 3-4 hrs, if the synthesis or modification of other factors is required. A number of mechanisms appear to be involved in transcriptional regulation of TGF-βs. The induction of α(2) I collagen gene expression by TGF-β1 is mediated by a nuclear factor-1 (NF-1) binding site in the collagen promoter (Rossi *et al.*, 1988). However, the autoinduction of TGF-β1 and coinduction of c-*jun* appear to be mediated by AP-1 binding sites (Kim *et al.*, 1990). The negative transcriptional response of TGF-β on transin expression appears to be mediated by a Fos-containing protein complex that binds to a cis-acting element, termed TGF-β inhibitory element (Kerr *et al.*, 1990). Post transcriptional effects on mRNA stability may also contribute to a change in mRNA expression (Raghow *et al.*, 1987; Penttinen *et al.*, 1988; Coffey *et al.*, 1988b).

The signal transduction of TGF-βs from the receptor through the cytoplasm of the nucleus affecting gene transcription is poorly understood. The cloning and characterization of TGF-β receptors will be important for a better understanding of this process. Additionally, the target genes that are directly transcriptionally modulated by TGF-βs may provide valuable information. Intracellular factors that coordinately regulate the expression of TGF-β modulated genes in a similar fashion as TGF-βs themselves, possibly function as mediators in the signal transduction pathway of TGF-βs.

VI. Expression of TGF-βs during Mammalian Embryogenesis

The *in vitro* effects of TGF-βs (including chemotaxis, regulation of growth and differentiation and stimulation of ECM), their expression during embryogenesis (see discussion below) and the presence of TGF-β receptors and ability of embryonic cells to respond to TGF-βs (Rizzino, 1987; Hill *et al.*, 1987; Centrella *et al.*, 1987) suggest that TGF-βs are likely to play a key role in the control of differentiation and cell specification in embryonic development.

TGF-β1 expression is found in mouse pre-implantation blastocysts after fertilization (Rappolee *et al.*, 1988) and differentiated embryonal carcinoma (EC) cells and embryonic stem (ES) cells (which share characteristics with pluripotent cells of the inner cell mass in the blastula) express TGF-β1 and TGF-β2 (van Zoelen *et al.*, 1989; Weima *et al.*, 1989; Mummery *et al.*, 1990). Northern blot analysis of RNA prepared from mouse embryos between 10.5 and 17.5 days post coitum (p.c) showed that TGF-β1 expression increases over the 7 day period. The total TGF-β2 expression increased, although multiple TGF-β2 mRNA transcripts show differential regulation and TGF-β3 expression increases between days 10.5 and 15.5 p.c. and TGF-β3 expression decreases slightly at day 17.5 (Miller *et al.*, 1989, a,b).

Measuring mRNA expression by *in situ* hybridization during mouse and human embryogenesis show that TGF-βs are expressed highly localized with different temporal and spatial patterns, though the expression of each different isoform occasionally overlap (Sandberg *et al.*, 1988 a,b; Lehnert and Akhurst, 1988; Wilcox and Derynck, 1988; Pelton *et al.*, 1989; Pelton *et al.*, 1990; Gatherer *et al.*, 1990). Comparing the expression of the three TGF-β isoforms during the first trimester of human embryogenesis show that TGF-β1 is expressed in hematopoietic, endothelial and osteogenic tissues. TGF-β2 and TGF-β3 are expressed in a wide variety of mesenchymal tissues including areas of chondrogenic activity. TGF-β2 expression is also expressed in several epithelia and in neutral nervous system (Gatherer *et al.*, 1990). Similar transcript distributions are found in murine embryos, suggesting a conservation of the gene regulatory elements for TGF-βs at these early stages.

Comparing *in situ* hybridization of TGF-β1 with immunolocalization studies in mice with a TGF-β1 antibody (Ellingsworth *et al.*, 1986; Heine *et al.*, 1987) both autocrine and paracrine modes of action have been proposed for TGF-β1 (Lehnert and Akhurst, 1988). High levels of TGF-β1 RNA and TGF-β1 protein were found in tissues undergoing osteogenesis, hematopoiesis, vascularization and angiogenesis. Based upon the known biological activities of TGF-β1, a possible autocrine mechanism of action of TGF-β1 is proposed for these tissues. In the more differentiated tissue, with both epithelial and mesenchymal tissues, like the developing hair follicles of the snout, the developing tooth bud, the submandibular salivary gland and the secondary palate, *in situ* hybridization detect TGF-β1 in the epithelial cells, (Lehnert and Akhurst, 1988), whereas TGF-β1 protein is detected in the underlying mesenchymal layer (Heine *et al.*, 1987). A paracrine mechanism of action is proposed in which epithelial synthesized TGF-β1 localizes in the mesenchyme, possibly to stimulate extracellular matrix deposition and to modulate mesenchymal condensation. The absence of TGF-β1 protein in a tissue expressing high levels of mRNA may be explained that the TGF-β1 is synthesized in a latent form and is not recognized by the antibody. (Alternatively, the TGF-β1 antibody may recognize other members of the TGF-β family). The roles of the various isoforms of TGF-β during embryogenesis will be investigated when isoform specific antibodies and antibodies that can distinguish between latent and active form, become available.

VII. Clinical Implications of TGF-βs

Pathological role of TGF-β in disease

TGF-βs are potent growth inhibitors for many cells, including epithelial and lymphoid cells. When a cell becomes refractory to growth inhibition by TGF-βs, this may lead to malignant cell growth (Sporn and Roberts, 1985). Indeed, TGF-βs are not growth inhibitory for particular transformed cells, including transformed keratinocytes, leukemia cells and retinoblastoma cells (Shipley *et al.*, 1986; Keller *et al.*, 1989, Kimchi *et al.*, 1988). Pharmacological agents that enhance the cellular secretion of TGF-βs in a premalignant state, before the cells have become insensitive to TGF-βs, may be useful agents for chemoprevention of cancer. Retinoic acid in keratinocytes and tamoxifen in estrogen receptor positive (ER+) breast cancer cells are able to upregulate the expression TGF-β (Glick *et al.*, 1990; Arrick *et al.*, 1990). Interestingly, both agents have successfully been used in animals to prevent skin and breast cancer (Boone *et al.*, 1990; Fentiman, 1990). This chemoprevention approach may, however, only be useful when it will be possible to diagnose patients at high risk for these type of cancers in a premalignant state.

TGF-βs are potent inducers of extracellular matrix formation *in vitro* and *in vivo*. Therefore, elevated expression and activity of TGF-βs could possibly lead to excessive connective tissue deposition as seen in fibrotic disorders, including keloids, cirrhoris, pulmonary fibrosis, and scleroderma. Elevated levels of TGF-β expression have been observed in pulmonary fibrosis (Raghow *et al.*, 1989; Khalil *et al.*, 1989) and in hepatic fibrosis (Czaja *et al.*, 1989). The TGF-β2 levels correlated with the severity of intraocular fibrosis (Connor *et al.*, 1989). These results suggest a possible role for TGF-βs in the pathogenesis of diseases in which fibrosis is a factor and the possibility of therapeutic intervention using inhibitors of the action of TGF-βs. Indeed, recent data suggest that antagonists of TGF-β1 may be useful in the prevention of glomerulonephritis, a kidney disease in which excess extracellular matrix deposition results in impaired filtration and loss of protein retention. TGF-β1 is directly implicated in the matrix deposition associated with this disease as antibodies directed against TGF-β1 were able to reverse this experimentally induced glomerulonephritis in rats (Border *et al.*, 1990).

Therapeutic opportunities of TGF-βs

TGF-βs are regulators of proliferation, differentiation and other cell functions in a wide variety of cell types. Physiologically TGF-βs are thought to play an important role in embryogenesis, tissue repair, immunomodulation and inflammation. This suggests a number of potential therapeutic applications of TGF-βs (Sporn *et al.*, 1989) which are described below. Until recently, little was known about the *in vivo* effects of TGF-β as animals studies with TGF-β, extracted from tissues, have been very expensive to perform. However, the large amounts of protein necessary for animal and clinical studies have become available with the recent recombinant expression of TGF-βs. Multiple animal studies using this material have already shown promising results (Sporn and Roberts, 1989).

TGF-βs have been shown to stimulate wound repair in a variety of animal models. In a model for deep wounds using wire mesh chambers inplanted subcuteneously in rats, TGF-β1 stimulated the formation of granulation tissue (Sprugel *et al.*, 1988: Sporn *et al.*, 1983). TGF-β1 also accelerated the healing and increased tensile strength of incisonal wounds in rats (Mustoe *et al.*, 1987), which are analogous to surgical wounds. TGF-β1 was very effective in impaired healing models induced by adriamycin (Lawrence *et al.*, 1986). This may have important clinical relevance as a wide variety of clinical conditions exist, where patients exhibit significant impaired wound healing including

patients undergoing systemic drug therapy on glucocorticoids or cancer chemotherapy and patients with vascular insufficiencies such as diabetic ulcers or sickle cell disease (ten Dijke and Iwata, 1989).

Several observations indicate that TGF-βs could also possibly enhance bone repair. TGF-β1 injected under the periosteum of uninjured newborn rat femurs initiated intramembranous bone formation (Joyce *et al.*, 1990a,b). Injection of TGF-β1 onto the periostea of parental bone of neonatal rats stimulated the formation of periosteal woven bone (Noda and Camilliere, 1989).

In vitro TGF-βs have potent immunosuppressive and anti-inflammatory effects. *In vivo* administration of TGF-βs can suppress myeloid and lymphoid cell proliferation (Goey *et al.*, 1989; Fontana *et al.*, 1989). Therapeutically, the immunosuppressive activity of TGF-β may be useful for treatment of autoimmune diseases and in preventing graft versus host disease. Systemic administration of TGF-β to mice was shown to prolong the heart graft survival significantly (Palladino *et al.*, 1990). Additionally, the potent reversible growth inhibition of hematopoietic cells by TGF-β may be used to protect these normal cells during chemo-radiotherapy of neoplastic disease. The action of TGF-βs as anti-inflammatory agents was demonstrated when TGF-β1 and TGF-β2 increased survival of rats injected with pathogenic *E. coli*, suggesting that TGF-βs may prevent septic shock due to bacterial infections. Possibly, the TGF-βs are decreasing the production of TNF-α, which is a key mediator in septic shock (Palladino *et al.*, 1990).

Clearly, TGF-βs hold a therapeutic promise for many clinical conditions and may provide solutions to illnesses that are currently not amenable to effective treatment. However, for appropriate use of TGF-βs as therapeutic agents, further understanding of the molecular and cellular mechanisms that determine their (patho)-physiological role and pharmacological activity is essential.

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CHAPTER FOUR

Identification of Another Member of the Transforming Growth Factor Type β Family

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Identification of another member of the transforming growth factor type β gene family

(tissue-derived growth inhibitor/transforming growth factor type β3/cDNA cloning)

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ABSTRACT **We report here the complete amino acid sequence of another member of the type β transforming growth factor gene family, deduced from the nucleotide sequence of three overlapping cDNA clones. The C-terminal 112 amino acids share ≈80% sequence identity with type β1 and β2 transforming growth factors, with many of the remaining differences being conservative substitutions. By analogy to type β1 and type β2 transforming growth factors, we predict the protein to be synthesized as a 412 amino acid precursor that undergoes proteolytic cleavage to produce the mature polypeptide.**

In contrast to the rapid accumulation of information over the past few years regarding positive regulators of cell growth (1–3), only limited progress has been made in efforts to identify regulatory factors that maintain tissue homeostasis by means of growth inhibition. Two growth inhibitors that have been the focus of considerable attention in recent years are transforming growth factor types β1 and β2 (TGF-β1 and TGF-β2).

TGF-β1 was first identified by its ability to induce colony formation of certain rodent cell lines in soft agar (4, 5). Subsequently, however, TGF-β1 was found to act as a potent growth inhibitor of a variety of cell lines (6). Both TGF-β1 and -β2 are synthesized as precursors that undergo dimerization and proteolytic cleavage to produce the biologically active polypeptide. In their mature forms, which consist of the C-terminal 112 amino acids, TGF-β1 and -β2 exhibit 71% sequence identity. TGF-βs have been proposed to effect tissue repair, wound healing, bone formation, and embryonic development (7–9). Abnormalities in TGF-β function have been implicated in both immunosuppression and cellular transformation (8).

In analyzing human tissues and cell lines as potential sources of tumor inhibitory factors, we discovered that extracts from umbilical cord contain multiple growth inhibitory activities, which we termed tissue-derived growth inhibitors (TGIs) (10). One of these TGI activities was purified to homogeneity and appeared to be indistinguishable from TGF-β1, whereas the initial characterization of a second TGI revealed a distinct but closely related protein. Conventional purification of this activity provided only limited quantities of material for analysis. An alternative strategy was to isolate the cDNA sequences for this TGI† using TGF-β1 cDNA as a probe, predicting that the inhibitor could possess sequence similarity to TGF-β1.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides, synthesized by the phosphoramidite method with an Applied Biosystems (Foster City, CA) model 380 A synthesizer used as specified by the manufacturer, were purified by polyacrylamide gel electrophoresis and desalted on a Waters SEP-PAK C_{18} cartridge.

DNA Labeling and Hybridization Methods. Nick-translation of DNA restriction fragments and filter hybridizations were done as described (11). The specific activities of the probes were $>2.5 \times 10^8$ cpm/μg. Oligonucleotides were phosphorylated using [γ-^{32}P]ATP and T4 polynucleotide kinase (12). Specific activities were $>2 \times 10^6$ cpm/pmol. Filters were prehybridized for 2 hr in 2.5 × SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/10× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% $NaDodSO_4$, followed by 2 hr at 65°C in hybridization mixture [5× SSC/50 mM sodium pyrophosphate/10× Denhardt's solution/10% dextran sulfate/single-stranded DNA at 250 μg/ml/7% (wt/vol) $NaDodSO_4$]. Hybridization with the labeled oligonucleotide probes was performed overnight in hybridization mixture at 45°C. The filters were washed with 3× SSC/10× Denhardt's solution/0.5% $NaDodSO_4$ and exposed to Kodak XAR-2 film at −70°C with a DuPont Lightning Plus intensifying screen.

Construction of a cDNA Library from the Human A673 Rhabdomyosarcoma Cell Line. Poly(A)$^+$ RNA was prepared from A673 cells (CRL 1598, American Type Culture Collection) by twice passing total RNA, isolated by the lithium chloride method (13), over an oligo(dT)-cellulose column (14). Before cDNA synthesis, the RNA was treated with RNase-free DNase (Promega Biotec, Madison, WI) in the presence of the ribonuclease inhibitor RNasin (Promega Biotec). Starting with 5 μg of poly(A)$^+$ RNA, a random-primed cDNA library of approximately 2×10^6 clones was constructed in λgt10, with the Amersham cDNA Synthesis System Plus according to the manufacturer's procedures [which are based on the method of Gubler and Hoffman (15)].

Subcloning and DNA Sequencing. *Eco*RI inserts from positive clones were subcloned in both orientations either in M13 sequencing vectors or in pGEM vectors (Promega Biotec) from which deletion clones were generated by Exonuclease III digestion (16). Sequencing was done according to the dideoxynucleotide chain-termination method (17).

RNA Analysis. Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography of total RNA isolated from umbilical cord and various cell lines by the lithium chloride method (13). Ten micrograms of poly(A)$^+$ RNA were fractionated on a 1% agarose gel in the presence of formaldehyde and then transferred to nitrocellulose (12). Radioactive labeling of the probes was done by the random-priming method (18). Blots

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Abbreviation: TGI, tissue-derived growth inhibitor; TGF-β1, -β2, and -β3, transforming growth factor type β1, type β2, and type β3, respectively.

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03241).

were prehybridized in 50% (vol/vol) formamide/50 mM sodium phosphate, pH 6.5/5× SSC/2.5× Denhardt's solution/salmon sperm DNA at 250 μg/ml/poly(A) at 10 μg/ml for 4 hr at 55°C. Hybridization was performed in prehybridization mix/50% (wt/vol) dextran sulfate, 4:1, for 12 hr at 55°C. The filters were washed in SSC buffered with 20 mM sodium phosphate, pH 6.5/1 mM EDTA/0.1% $NaDodSO_4$. Six 20-min washes were carried out at 65°C, stepwise lowering the concentration of SSC from 0.3× to 0.1× to 0.03×. Filters were exposed to Kodak XAR-2 film with a DuPont Lightning Plus intensifying screen at −70°C.

RESULTS AND DISCUSSION

Identification and Sequence Analysis of a TGI cDNA. Based on the published sequence of TGF-β1 cDNA (19), we synthesized a 25-mer oligonucleotide (5′ CGGCACCGCC-GAGCCCTGGACACCA 3′, bases 826–851 of the coding region). This probe was then used to identify a TGF-β1 cDNA clone in a commercial λgt11 human placenta library (Clontech, Palo Alto, CA). By restriction mapping and partial sequence analysis, the clone was shown to contain the complete coding sequence for the 390-amino acid TGF-β1 precursor but to lack parts of the untranslated regions [439 base pairs (bp) from the 5′ end and ≈200 bp from the 3′ end, data not shown). A *Pvu* II–*Pvu* II fragment of the TGF-β1 cDNA was then used as a hybridization probe for a Southern blot (20) of human umbilical cord genomic DNA, digested with *Eco*RI, *Hin*dIII, or *Sst* I (Fig. 1). In each digest two bands were apparent at low stringency (2.5× SSC, 65°C, lanes 1, 3, and 5); when the stringency was increased, only one hybridizing band remained visible (0.01× SSC, 65°C, lanes 2, 4, and 6). We presumed that the strongly hybridizing band corresponded to TGF-β1, whereas the detection of a weakly hybridizing second band suggested the presence of a related gene. Both genes were cloned by screening a genomic phage library constructed from the human K-562 chronic myelogenous leukemia cell line according to the procedures of Grosveld *et al.* (21). By varying the wash stringency, the TGF-β sequences could be distinguished from the TGF-β-related sequences. Restriction mapping and partial sequence analysis revealed that clones hybridizing at high stringency were derived from the gene encoding TGF-β1, whereas a clone that hybridized only at low stringency appeared to be derived from a related gene. We have termed this DNA sequence TGF-β3 in the remainder of the text (see discussion below).

To obtain a repeat-free probe for TGF-β3, restriction fragments of the genomic clone were separated by electrophoresis, transferred to nitrocellulose, and then hybridized with either TGF-β1 cDNA or total human genomic DNA. A *Bam*HI–*Taq* I fragment was found to hybridize specifically to

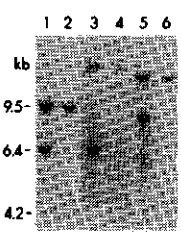


FIG. 1. Southern blot analysis of umbilical cord genomic DNA digested with *Eco*RI (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), and *Sst* I (lanes 5 and 6) and hybridized with a cDNA fragment of TGF-β1. Lanes 1, 3, and 5: low-stringency wash. Lanes 2, 4, and 6: high-stringency wash.

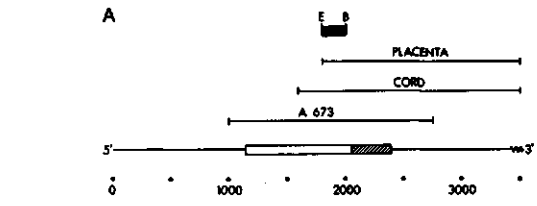
TGF-β1 cDNA, indicating the presence of coding sequence and the absence of repetitive DNA elements. This repeat-free probe was used to screen a λgt11 human placenta cDNA library (Clontech, 1.2×10^6 independent clones) resulting in the isolation of a 1.7-kilobase (kb) cDNA clone (Fig. 2). Screening of a λgt11 human umbilical cord cDNA library (Clontech, 1.5×10^6 independent clones) with a 5′ *Eco*RI–*Bgl* II restriction fragment (Fig. 2, indicated as E–B), derived from the placenta cDNA clone, resulted in the isolation of a 1.9-kb cDNA. The nucleotide sequences of these two cDNA clones confirmed that both were derived from the same gene as the genomic TGF-β3 clone. On RNA analysis, however, the TGF-β3 mRNA was found to be ≈3.5 kb, indicating that we had not obtained a full-length cDNA.

To obtain a full-length clone, mRNA was isolated from A673 cells, and a cDNA library was prepared by random priming. Approximately 0.7×10^6 unamplified cDNA clones were screened with a 25-mer oligonucleotide probe (5′ ATATAGCGCTGTTTGGCAATGTGCT 3′, corresponding to a sequence near the 5′ end of the 1.9-kb cDNA clone), and a single positive clone containing a 1.7-kb insert was identified.

Analysis of the three overlapping cDNAs (Fig. 2) revealed a sequence of 2529 bp, with the largest open reading frame being 1236 bases. We found no sequence differences in the overlapping regions and conclude that all three clones were derived from transcripts of the same gene. Our sequence contains a complete 3′ untranslated region of 1031 bp with a polyadenylylation signal 25 bp upstream from the poly(A) tract. The 5′ untranslated region comprises 262 bp and lacks ≈1 kb, as judged from the size of the mRNA estimated by RNA analysis. The predicted amino acid sequence of TGF-β3 shows extensive similarity to TGF-β1 and -β2 (19, 22). Failure of the TGF-β1 probe to detect TGF-β2 in the original Southern hybridization (Fig. 1) can be accounted for by the fact that the *Pvu* II–*Pvu* II TGF-β1 cDNA fragment shows 75% similarity with TGF-β3 but only 65% similarity with TGF-β2. Comparing TGF-β3 with the GenBank 48.0 nucleotide sequence data base‡ using the FASTN program (23) revealed no significant similarity with genes other than known members (see below) of the TGF-β family.

A Comparison of TGF-β1, -β2, and -β3. TGF-β1 and TGF-β2 are produced in precursor forms of 390 and 414 amino acid residues, respectively (19, 22). The cDNA sequence we have obtained for TGF-β3 (Fig. 2*b*) contains an open reading frame coding for 412 amino acids, with the first ATG preceded by a stop codon, 162 nucleotides upstream. As found with TGF-β1 (19) and -β2 (22), the predicted initiating codon for TGF-β3 does not form part of a Kozak consensus sequence (24). Interestingly, six nucleotides downstream there is a second ATG, with an adenine at position −3, which aligns with the initiating codon in TGF-β2. Homodimers of the C-terminal 112 residues of TGF-β1 and -β2 represent the biologically active forms of these proteins. Preceding the site of cleavage to their mature forms, TGF-β1 and -β2 have stretches of four and five basic residues, respectively. In TGF-β3 there are five basic residues preceding the predicted cleavage site (Fig. 3). The mature forms of TGF-β1 and -β2 share 80/112 identical residues. The corresponding 112 C-terminal amino acids of TGF-β3 exhibit 86/112 and 89/112 identical residues compared with TGF-β1 and -β2, respectively (Fig. 3). Many remaining differences represent conservative substitutions. All three proteins show a strict conservation of the nine cysteine residues in this region (19, 22). The N-terminal domain of the precursor form of TGF-β3 exhibits ≈35%

‡EMBL/GenBank Genetic Sequence Database (1987) GenBank (IntelliGenetics, Mountain View, CA), Tape Release 48.0.



B

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CCAGCAAAAGCCTGTTTAGACACATGGACAAGAATCCCAGCGCTACAAGGCACACAGTCCGCTTCTTCGTCCTCAGGGTTGCCAGCGCTTCCTGGAAGTCC
        10        20        30        40        50        60        70        80        90       100
TGAAGCTCTCGCAGTGCAGTGAGTTCATGCACCTTCTTGCCAAGCCTCAGTCTTTGGGATCTGGGGAGGCCGCCTGGTATTTCCTCCCTCCTTCTGCACGT
       110       120       130       140       150       160       170       180       190       200
                                                                 M  K  H  W  L  Q  R  A  L  V  V  L  A
CTGCTGGGGCTCTCTTCCTCTCCAGGCCTTGCCGTCCCCCTGGCCTCTCTTCCCAGCTCACACATGAAGATGCACTTGCAAAGGGCTCTGGTGGTCCTGGC
       210       220       230       240       250       260       270       280       290       300
  L  L  N  F  A  I  V  S  L  S  L  S  T  C  T  F  L  D  F  G  H  I  K  K  K  R  V  E  A  I  R  G  Q
CCTGCTGAACTTTGCCACAGGTCAGCCTCTCTCTGTCCACTTGCACCACCTTGGACTTCGGCCACATCAAGAAGAAGAGGGTGGAAGCCATTAGGGGACAG
       310       320       330       340       350       360       370       380       390       400
  I  L  S  K  L  R  L  Y  S  P  P  E  P  T  V  H  T  H  V  P  Y  Q  V  L  A  L  Y  N  S  T  R  E  L
ATCTTGAGCAAGCTCAGGCTCACCAGCCCCCCCTGAGCCAACGGTGATGACCCACGTCCCCTATCAGGTCCTGGCCCTTTACAACAGCACCCGGGAGCTGC
       410       420       430       440       450       460       470       480       490       500
  L  E  E  M  N  G  E  R  E  E  G  C  T  Q  E  N  T  E  S  E  Y  Y  A  K  E  I  N  K  F  D  M  I  Q  G
TGGAGGAGAATGCATGGGGAGAGGGAGGAAGGCTGCACCCAGGAAAACACCGAGTCGGAATACTATGCCAAAGAAATCCATAAATTCGACATGATCCAGGG
       510       520       530       540       550       560       570       580       590       600
  L  A  E  M  N  E  L  A  V  C  P  K  G  I  T  S  K  V  F  R  F  N  V  S  S  V  E  K  N  R  T  N  L
GCTGGCGGAGCACAACGAACTGGCCTGTCTGCCCTAAAGGAATTACCTCCAAGGTTTTCCGCTTCAATGTGTCCTCAGTGGAGAAAAATAGAACCAACCTA
       610       620       630       640       650       660       670       680       690       700
  F  R  A  E  F  R  V  L  R  V  P  N  P  S  S  K  R  N  E  Q  R  I  E  L  F  Q  I  L  R  P  D  E  H
TTCCGAGCAGAATTCCGGCTCTTGCGGGTGCCCAACCCCAGCTCTAAGCGGAATGAGCAGAGGATCGAGCTCTTCCAGATCCTTCGGCCAGATGAGCACA
       710       720       730       740       750       760       770       780       790       800
  I  A  Q  G  Y  I  G  K  N  L  P  T  R  G  T  A  E  W  L  S  F  D  V  T  D  T  V  R  E  W  L  L
ATTGCCAAGCAGGCGTATATCGGTGGCAAGAATCTGCCCACACGGGGCACTGCCGAGTGGCTGTCCTTTGATGTCACTGACACTGTGCGTGAGTGGCTGTT
       810       820       830       840       850       860       870       880       890       900
  R  R  E  S  N  L  G  L  E  I  S  I  H  C  P  C  N  T  F  Q  P  N  G  D  I  L  E  N  I  R  E  V  M
GAGAAGAGAGTCCAACTTAGGTCTAGAAATCAGCATTCACTGTCCATGTCACACCTTCAGCCCAATGGAGATATCCTGGAAAACATTCACGAGGTGATG
       910       920       930       940       950       960       970       980       990      1000
  E  I  K  F  K  G  V  D  N  E  D  D  H  G  R  G  D  L  G  R  L  K  K  Q  K  D  H  H  H  P  H  L  I
GAAATCAAATTCAAAGGCGTGGACAATGAGGATGACCATGGCCGTGGAGATCTGGGGCGCCTCAAGAAGCAGAAGGATCACCACAACCCTCATCTAATCC
      1010      1020      1030      1040      1050      1060  *   1070      1080      1090      1100
  L  N  E  P  P  N  R  L  P  K  P  G  Q  G  G  P  R  K  R  A  L  D  T  N  Y  C  F  R  N  L  E  E
TCATGATGATTCCCCCACACCGGCTCGACAACCCGGGCCAGGGGGGTCAGAGGAAGAAGCGGGCTTTGGACACCAATTACTGCTTCCGCAACTTGGAGGA
      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
  H  C  C  V  R  P  L  Y  I  D  F  R  Q  D  L  G  W  K  W  V  H  E  P  K  G  Y  Y  A  N  F  C  S  G
GAACTGCTGTGTGCGCCCCCTCTACATTGACTTCCGACAGGATCTGGGCTGGAAGTGGGTCCATGAACCTAAGGGCTACTATGCCAACTTCTGCTCAGGC
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
  P  C  P  Y  L  R  S  D  T  N  S  T  V  L  G  L  Y  N  T  L  H  P  E  A  S  A  S  P  C  C  V
CCTTGCCCATACCTCCGAGATGCAGACAACCCACAGCACGGTGCTGGGACTGTACAACACTCTGAACCCTGAAGCATCTGCCTCGCCTGCTGCGTGC
      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
  P  Q  D  L  E  P  L  T  I  L  Y  Y  V  G  R  T  P  K  V  E  Q  L  S  N  M  V  V  K  S  C  K  C  S
CCCAGGACCTGGAGCCCCTGACCATCCTGTACTATGTTGGGAGGACCCCCAAAGTGGAGCAGCTCTCCAACATGGTGGTGAAGTCTTGTAAATGTAGCTG
      1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
AGACCCCACGTGCGACAGAGAGAGGGGAGAGAACCACCACTGCCTGACTGCCCGCTCCTCGGGAAACACACAAGCAACAAACCTCACTGAGAGGCCTG
      1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
GAGCCCACAACCTTCGGCTCGGGCAAATGGCTGACATGGAGGTTTCCTTTGGAACATTCTTTCTTGCTGGCTCTGAGAATCACGGTGGTAAAGAAAG
      1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
TGTGGGTTTGGTTAGAGGAAGGCTGAACTCTTCAGACACACACAGACTTTCTGTGACGCACACAGAGGGGATGGGGATAGAGGAAAGGGATGGTAAGTTGA
      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
GATGTTGTGTGGCAATGGGATTTGGGCTACCCTAAAGGGAGAAGGAAGGGCAGAGAATGGCTGGGTCAGGGCCAGACTGGAAGACACTTCAGATCTGAGG
      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
TTGGATTTGCTCATTGCTGTACCACATCTGCTCTAGGGAATCTGGATTATGTTATACAAGGCAAGCATTTTTTTTTTTTTTAAAGACAGGTTACGAAGA
      1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
CAAAGTCCCAGAATTGTATCTCATACTGTCTGGGATTAAGGGCAAATCTATTACTTTTGCAAACTGTCCTCTACATCAATTAACATCGTGGGTCACTACA
      2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
GGGAGAAAATCCAGGTCATGCAGTTTCCTGGCCCATCAACTGTATTGGGCCTTTTGGATATGCTGAACGCACGAAGAAAGGGTGGAAATCAACCCTCTCCTG
      2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
TCTGCCTCTGGGTCCCTCCTCTCACCCTCTCCCTCGATCATATTTCCCCTTGGACACTTGGTTACACGCCTTCCAGGTCAGGATGCACATTTCTGGATTGT
      2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
GGTTCCATGCAGGGTTGGGGCATTATGGGTTCTTCCCCCACTTCCCCTCCAAGACCCTGTGTTCATTTGGTGTTCCTGGAAGCAGGTGCGACAACATGTG
      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
AGGCATTCGGGGAAGCTCGACATGTGCCACACAGTGACTTGGCCCCAGACGCATAGACTGAGGTATAAAGACAAGTATGAATATTACTCTCAAAATCTTT
      2410      2420      2430      2440      2450      2460      2470      2480      2490      2500
GTATAAATAAATATTTTGGGGCATCCTG poly(A)
      2510      2520
```

FIG. 2. (*A*) Schematic diagram of TGF-β3 mRNA with the coding sequence boxed. The relative extension of the cDNAs obtained from placenta (1.7 kb), umbilical cord (1.9 kb), and A673 (1.7 kb) libraries is indicated. The dashed part of the box represents the C-terminal region showing the greatest similarity to TGF-β1 and -β2. The 5′ *Eco*RI–*Bgl* II restriction fragment of the placental cDNA used to screen the umbilical cord library is indicated by a thick bar (E–B). (*B*) Nucleotide sequence and deduced amino acid sequence of a TGF-β3 cDNA, determined from three overlapping cDNA clones. Putative glycosylation sites and the polyadenylylation signal are underlined. The predicted start of the mature TGF-β3 is marked by an asterisk at position 1164.

similarity with TGF-β1 and 45% similarity with TGF-β2. By comparison, the corresponding sequences of the TGF-β1 and -β2 precursors have 33% sequence similarity (Fig. 3). A homology matrix plot clearly illustrates the greater similarity between TGF-β3 and -β2 compared with TGF-β1 (Fig. 3). Four potential glycosylation sites are contained in the N-terminal part of TGF-β3, one of which is conserved in all three proteins. All three proteins also possess hydrophobic N termini, which probably represent presecretory signal peptide sequences (25). Interestingly, both TGF-β1 and TGF-β3 (but not TGF-β2) contain the fibronectin-binding sequence Arg-Gly-Asp (26). Given the known effects of TGF-β1 on the extracellular matrix and wound healing (8), it is tempting to speculate that TGF-β3 may also have therapeutic applications in this area.

Expression of TGF-β3 mRNA. The availability of a TGF-β3 cDNA clone allowed us to investigate expression of the gene in different human cell types. Hybridizing mRNA was found to be present in A673 (a rhabdomyosarcoma), A549 (a lung adenocarcinoma), and A-498 (a kidney carcinoma); expression of TGF-β3 was found to be lower by at least a factor of 50 than that of TGF-β1. In all three cell lines, the TGF-β3 mRNA migrated as a species of about 3.5 kb (Fig. 4). A faintly hybridizing band was found at 4.2 kb, which was also seen

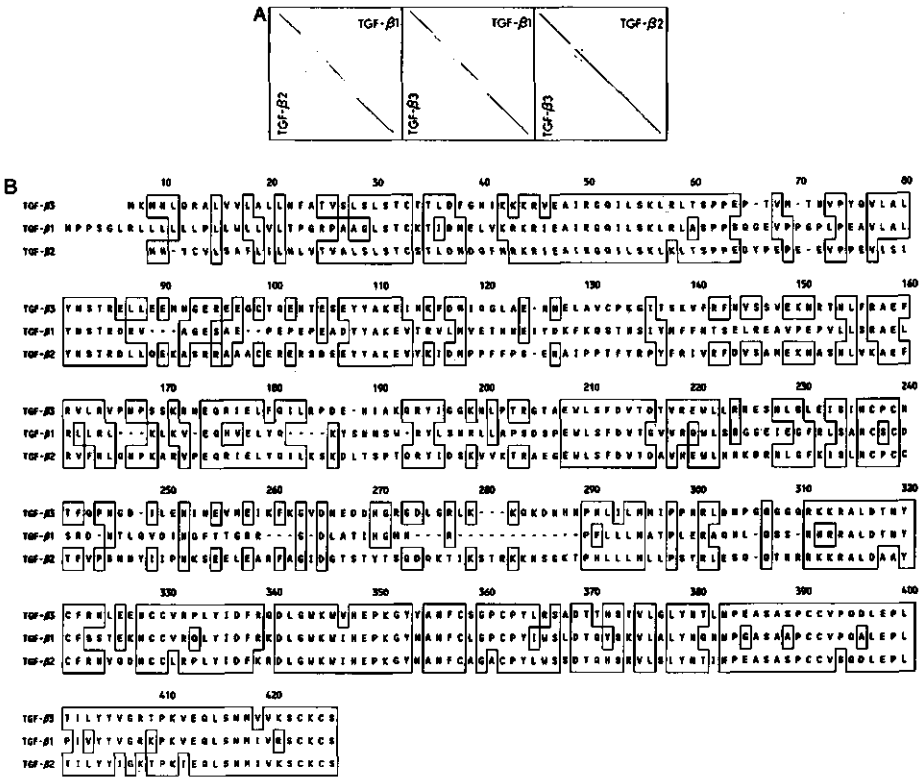

FIG. 3. Homology of TGF-βs. (*A*) Homology matrix plots of TGF-β amino acid sequences. Stretches of 17 amino acids sharing at least 50% identity between two proteins were scored as homologous. (*B*) Comparison of the deduced amino acid sequences of TGF-β3, TGF-β1, and TGF-β2 precursors. One-letter code for amino acids is used, and gaps have been introduced to maximize the similarities. Identical amino acids are boxed. The mature TGF-β amino acid sequences start at position 315.

with the TGF-β1 probe and may correspond to TGF-β2. Expression of TGF-β3 was not detected by the TGF-β1 probe in the experiment, shown in Fig. 4*A*, due to the short exposure. Interestingly, umbilical cord, which we had shown previously to contain multiple TGF-β-like activities, expresses the highest level of TGF-β3 mRNA that we have seen to date (Fig. 4). Peptide antisera to TGF-β3 confirm that the protein is present in umbilical cord extracts in fractions containing growth inhibitory activity (K.K.I. and S. I. Rayter, unpublished data).

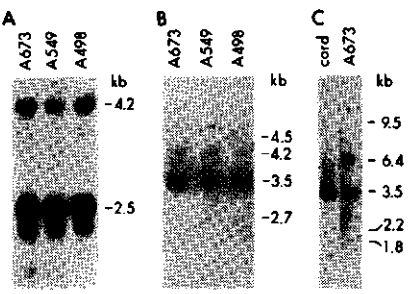

FIG. 4. RNA blot analysis. (*A*) RNA blot analysis of mRNA from A673, A549, and A-498 cell lines using a TGF-β1 probe containing the complete coding region. Exposure time, 14 hr. (*B*) RNA blot analysis of mRNA from A673, A549, and A-498 cell lines using the *EcoRI–BglII* placenta TGF-β3 probe (Fig. 2). Exposure time, 4.5 days. (*C*) RNA blot analysis of umbilical cord and A673 mRNA using the same probe as in *B*. Exposure time, 3 days.

The TGF-β Gene Family and Comments on Nomenclature. During the last few years TGF-β1 and -β2 have been shown to correspond to a number of previously described biological activities; these include the glioblastoma-derived T cell suppressor factor (22), cartilage-inducing factors A and B (27, 28), tumor growth inhibiting factor (29), and polyergin (originally termed BSC-1-derived growth inhibitor) (30). Clearly, TGF-βs are important in the regulation of cell growth. An inability of cells either to produce such inhibitory factors or to respond to them may play a critical role in transformation (7, 8), and members of the TGF-β family, therefore, could function as antioncogenes.

Recently, it has become apparent that the TGF-βs belong to an expanding family of genes, which not only act as potent regulators of cell growth but also affect cell differentiation. These genes include those that encode multiple forms of activin and inhibin, which regulate secretion of follicle-stimulating hormone (31, 32); the gene that encodes Mullerian

inhibiting substance, responsible for regression of the female reproductive tract during development of the male embryo (33); *Vg1*, which appears to be involved in mesoderm formation during *Xenopus* development (34); and the decapentaplegic gene of *Drosophila*, which is associated with dorsal–ventral determination during embryogenesis (35). Given the multiple activities exhibited by the TGF-β gene family, we predict that our cDNA clone may also encode a protein with pleiotropic biological functions, as opposed to being strictly another TGI. Therefore, neither the term TGF-β nor the term TGI is appropriate for this factor. Due to the current widespread use of the former nomenclature, however, we have chosen the term TGF-β3 for present designation. As has been proposed (30), a more appropriate nomenclature for this gene family is needed. One possibility would be the use of TGF as an abbreviation for tissue growth factor. Perhaps, however, such terminology should await a clearer definition of the *in vivo* physiology of these factors.

Note Added in Proof. Partial cDNA clones for the chicken TGF-β3 have been isolated recently by Jakowlew *et al.* (36). The predicted C-terminal 112 amino acids of chicken TGF-β3 share 111/112 residues sequence identity with the human protein described herein.

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CHAPTER FIVE

Transforming Growth Factor Type β3 Maps to Human Chromosome 14, Region q23-q24

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SHORT REPORT

Transforming growth factor type β3 maps to human chromosome 14, region q23-q24

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Type β transforming growth factors (TGF-βs) are polypeptides that act hormonally to control the proliferation and differentiation of multiple cell types. Recently, we reported the isolation of a cDNA encoding a new member of this gene family, which we have termed TGF-β3. Here we show by Southern analysis using a human probe specific for TGF-β3, the presence of a related single copy gene in a wide range of animal species. Chromosomal localization of the TGF-β3 gene was performed by Southern blot analysis of DNA prepared from 24 human-Chinese hamster somatic cell hybrids using a specific TGF-β3 cDNA probe. The human specific restriction fragments segregated only with human chromosome 14. For all other human chromosomes high discordancy scores were obtained. Using *in situ* hybridization of human metaphase chromosomes, the regional location could be identified. Hybridization of the TGF-β3 cDNA probe resulted in specific labeling of chromosome 14, bands q23–24.

Type β transforming growth factors (TGF-βs) are dimeric 25 kD peptides with widespread effects on proliferation, differentiation, embryogenesis and tissue repair (for recent reviews see Massague, 1987; Sporn *et al.*, 1987; ten Dijke *et al.*, 1988a). Three closely related homodimeric forms of TGF-β exist. The form that was described first, TGF-β1, is the predominant species in many cells and is particularly abundant in human platelets (Assoian *et al.*, 1983) and bovine bone (Seyedin *et al.*, 1986). A second form of TGF-β, TGF-β2, has been isolated from porcine platelets (Cheifetz *et al.*, 1987), bovine bone (Seyedin *et al.*, 1987) and conditioned media of a variety of cell types (Wrann *et al.*, 1987; Ikeda *et al.*, 1987; Hanks *et al.*, 1988). A rare iso-form containing one TGF-β1 and one TGF-β2 chain has been identified in porcine platelets and has been termed TGF-β1.2 (Cheifetz *et al.*, 1987). The physiological significance of this heterodimer, if any, is presently unknown. Although in most systems which have been examined, TGF-β1 and -β2 have been found to exhibit similar biological properties; distinct effects have also been observed in certain assays. For example, TGF-β2, but not TGF-β1, has been reported to induce *Xenopus* mesoderm induction (Rosa *et al.*, 1988), while TGF-β1, appears to be a 100 fold more potent growth inhibitor of hematopoietic stem cells (Ohta *et al.*, 1987).

During the last few years, it has become apparent that the TGF-βs belong to a rapidly growing family of polypeptides which share similar structural characteristics. Other mammalian members of the family include two forms of inhibin and three forms of activin, gonadal proteins that regulate pituitary secretion of follicle stimulating hormone (Ling *et al.*, 1986 and Mason *et al.*, 1985); and the Mullerian inhibiting substance or MIS, a protein which causes regression of the female rudiments in the developing male reproductive system (Cate *et al.*, 1986).

Recently, we reported the isolation of a cDNA clone for the third form of TGF-β, which we have termed TGF-β3 (ten Dijke *et al.*, 1988b). The C-terminal 112 amino acids of TGF-β3 share approximately 80% sequence identity with TGF-β1 and -β2. The C-terminal 112 amino acids of TGF-β1 are identical in human (Derynck *et al.*, 1985) porcine- (Derynck & Rhee, 1987), bovine- (Van Obberghen-Schilling *et al.*, 1987) and simian species (Sharples *et al.*, 1987) and differ by only one amino acid in the murine form (Derynck *et al.*, 1986). Furthermore, the human (de Martin *et al.*, 1987; Madisen *et al.*, 1988) and simian (Hanks *et al.*, 1988) forms of TGF-β2 have identical C-terminal 112 amino acid residues. To investigate the interspecies conservation of TGF-β3 in mammalian and avian species, a human 0.25 kb TGF-β3 specific probe was hybridized with genomic DNA of other species (see Figure 1). In each digest of monkey, rat, mouse, dog, cow, sheep and chicken DNA, TGF-β3 homologous sequences were identified; except for dogs, all species had one hybridizing EcoRI restriction fragment. Northern hybridization of mouse mRNA visualizes a TGF-β3 mRNA of similar length as the human counterpart (not shown). The TGF-β3 probe used in both of the hybridization experiments contains sequences corresponding to the N-terminal percursor domain that do not cross react with either the human TGF-β1 or -β2 sequences under these conditions. Following our publication of the human TGF-β3 sequence, the chicken TGF-β3 gene has also now been isolated (Jakowlew *et al.*, 1988). The predicted C-terminal 112 amino acids of human and chicken TGF-β3 share 111/112 residues sequence identity and have one conservative substitution. The intact percursors show 366/412 amino acid sequence identities. This high degree of evolutionary conservation amongst the TGF-βs suggests a fundamental role in normal cell physiology.

Using a specific [^{32}P]-labeled 0.45 kb TGF-β3 cDNA probe, the DNA from a panel of 24 human × Chinese hamster hybrids was screened for human TGF-β3 sequences. A representative Southern blot analysis is shown in Figure 2. By using this particular probe (which contains the DNA sequence of the probe used in Figure 1 and an extra 180 bp), two hybridizing EcoRI

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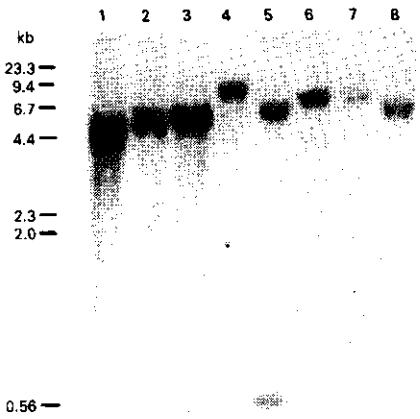

Figure 1 Southern blot analysis of EcoRI digested DNA (10 μg) from different animal species (liver tissue source except Human); (1) Human placenta; (2) Monkey; (3) Rat; (4) Mouse; (5) Dog; (6) Cow; (7) Sheep and (8) Chicken. Hybridization was performed with a [^{32}P]-labeled 5' EcoRI-SmaI restriction fragment derived from the 1.7 kb placenta cDNA clone (ten Dijke *et al.*, 1988b). Washing of the filter was performed at 2.5 × SSC, 65°C

fragments of 5.5 and 1.5 kb could be identified in human DNA (Figure 2, lane f). These fragments were easily distinguishable from the two bands hybridizing in Chinese hamster DNA (Figure 2, Lanes a–e), thereby allowing the identification and chromosomal localization of the human TGF-β3 gene. The overall results of this screening are presented in Table 1. The highest correlation is

Table 1 Relationship between the human TGF-β3 gene and human chromosomes in 24 human-Chinese hamster somatic cell hybrid clones

	No. hybrid clones with chromosome/TGF-β3 gene retention				
Chromosome	+/+	+/−	−/+	−/−	*% Discordance*
1	5	6	7	6	54
2	2	1	10	11	46
3	6	6	6	6	50
4	6	6	6	6	50
5	6	7	6	5	54
6	6	4	6	8	42
7	6	3	6	9	38
8	7	7	5	5	50
9	9	4	3	8	29
10	5	3	7	9	42
11	3	7	9	5	67
12	11	5	1	7	25
13	5	4	7	8	46
14	12	0	0	12	0
15	9	5	3	7	33
16	10	6	2	6	33
17	10	5	2	7	29
18	5	4	7	8	46
19	8	5	4	7	38
20	9	9	3	3	50
21	9	9	3	3	50
22	9	7	3	5	42
X	8	8	4	4	50

The symbols +/.. and −/.. indicate the presence and absence of the human chromosome. The symbols ../+ and ../− refer to the presence and absence of human TGF-β3 sequences as detected by Southern hybridization

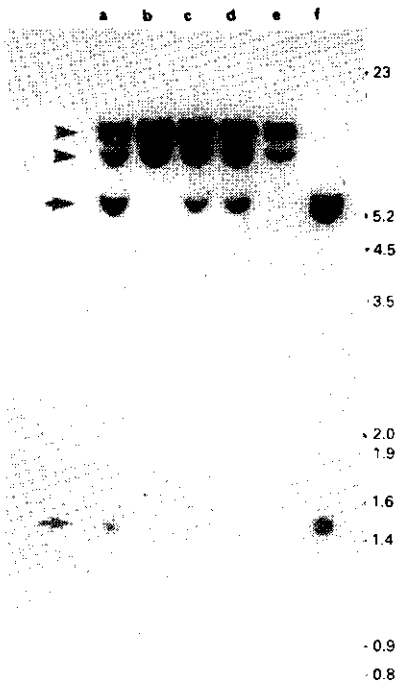

Figure 2 Southern blotting analysis of EcoRI digested DNA (15 μg) from human × Chinese hamster somatic cell hybrids (Lanes a–e) and control HeLa cells (Lane f). The panel of hybrid cell lines was generated by fusion of thymidine kinase-deficient (TK$^-$) or hypoxanthine phosphoribosyltransferase deficient (HPRT$^-$) Chinese hamster cells with leucocytes from various human donors. Details concerning their origin and initial characterization have been reported previously (Geurts van Kessel *et al.*, 1983). The same batches of cell were used for DNA extraction and cytogenetic characterization. The 5' EcoRI-SmaI restriction fragment derived from the 1.9 kb umbilical cord cDNA clone (ten Dijke *et al.*, 1988b) was used as a probe. Washing was performed at 0.3 × SSC at 65°C. The arrows indicate hybridization with the human TGF-β3 sequences, whereas arrow heads point at Chinese hamster hybridizing fragments. Hybrids a, c and d retained the human TGF-β3 sequences whereas the others did not (b and e)

found with chromosome 14. All 12 of the 24 hybrid cell lines which contained human chromosome 14 hybridized with the TGF-β3 probe (Table 1, +/+ column). No hybrids were found in the categories, in which chromosome 14 was present and TGF-β3 absent or vice versa (Table 1, +/− and −/+ column), in contrast to the findings with all other human chromosomes. This result is consistent with a single location for the TGF-β3 gene on chromosome 14.

In situ hybridization of the [^{3}H]-labeled TGF-β3 specific probe to metaphase chromosomes from a normal individual also resulted in the specific labeling of chromosome 14. Of 100 metaphase cells examined from this hybridization, 25 (25%) were labeled on region q2 of one or both copies of the chromosome 14 homologues. The distribution of labeled sites on this chromosome is illustrated in Figure 3; of 169 labeled sites observed, 37 (22%) were located on this chromosome.

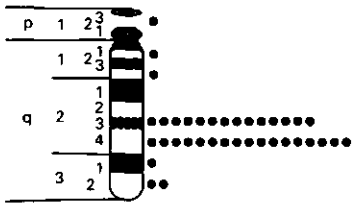

Figure 3 Distribution of labeled sites on chromosome 14 in 100 normal metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes that were hybridized with TGF-β3 specific cDNA probe. (Clone p5-1;5' EcoRI-EcoRI restriction fragment of the TGF-β3 cDNA containing nucleotides 0-715 (ten Dijke *et al.*, 1988b) subcloned into the pGem vector, Promega Biotec). The probe was prepared by nicktranslation of the entire plasmid with all four [^{3}H]-labeled deoxynucleoside triphosphates to a specific activity of $1.0–2.0 \times 10^8$ dpm μg^{-1}. *In situ* hybridization was performed as described previously (Le Beau *et al.*, 1984). Metaphase cells were hybridized at 2.0 and 4.0 ng of probe per ml of hybridization mixture. Autoradiographs were exposed for 11 days. Each dot indicates one labeled site observed in the corresponding band. Eighty four percent (31/37) of the labeled sites on chromosome 14 were located at q23-24; this cluster represented 18.3% of all labeled sites (31/169)

These sites were clustered at bands q23-24 and this cluster represented 18.3% (31/169) of all labeled sites (cumulative probability for the Poisson distribution is $\ll 0.0005$). Specific labeling of bands 14q23-24 was also observed in hybridizations in which we used two other TGF-β3 probes (data not given). All hybridization experiments were repeated at least twice and gave essentially identical results. It was noticeable that no hybridizations of the three TGF-β3-specific probes was observed at 19q13, the chromosomal localization of TGF-β1.

The sequence homology between members of the TGF-β gene family suggest a common evolutionary origin. Some related gene families are closely linked within the same chromosomal region; e.g., the globin gene family (Maniatis *et al.*, 1981), tumor necrosis factors (Nedwin *et al.*, 1985) and some of the interferons (Owerbach *et al.*, 1981). In contrast, TGF-β3, TGF-β1 and inhibin βA are localized on chromosome 14, 19 and 7, respectively (Fujii *et al.*, 1986; Barton *et al.*, 1987). This suggests that following amplification, these genes have dispersed and subsequently diverged in sequence.

The TGF-β3 gene maps to a region of chromosome 14 that contains a number of characterized genes, as well as the breakpoints of several recurring chromosomal abnormalities in human tumors. Genes that have been mapped to this region include *WARS* (tryptophanyl-tRNA synthetase), *COX4L1* (Cytochrome C oxidase subunit IV-like 1), *HSPA2* (heat shock protein 2, 70 kD subunit), *MTHFC* (5,10-methylene tetrahydrofolate cyclohydrolyase) as well as the *FOS* proto-oncogene (Ropers *et al.*, 1987). With respect to cancer-specific chromosomal abnormalities, deletions involving bands 14q22-24 have been observed in malignant lymphomas as well as in B-cell chronic lymphocytic leukemia, and a recurring translocation involving chromosomes 6 and 14, t(6; 14) (q21; q24) has been observed in ovarian carcinomas (Bloomfield *et al.*, 1987). Whether the TGF-β3 gene is involved in any of these malignancies remains to be determined.

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CHAPTER SIX

Recombinant Expression and Purification of Transforming Growth Factor-β3, a Potent Growth Regulator

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Recombinant Expression and Purification of Transforming Growth Factor-β3, a Potent Growth Regulator.

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Abstract

Recently, we identified a new member of the transforming growth factor-β (TGF-β) family, termed TGF-β3. Recombinant human TGF-β3 was expressed in Chinese Hamster Ovary cells by using dihydrofolate reductase gene amplification. By stepwise increases in the concentration of methotrexate, clones with amplified gene copy number and elevated expression levels were selected. Using antibodies against TGF-β3 peptides, it was shown that the conditioned media of transfected cells contained both the mature and precursor forms of TGF-β3. The TGF-β3 protein was purified to apparent homogeneity by ammonium sulfate precipitation, acetic acid/acetonitrile extraction of the pellet, followed by Biogel P60 gel filtration, immunoaffinity chromatography and reverse phase HPLC on C18. Biological activity was demonstrated by growth inhibition of Mv1Lu mink lung epithelial cells and the stimulation of rat NRK fibroblasts cells to form large colonies in soft agar cultures in the presence of epidermal growth factor. Additionally, TGF-β3 inhibited the growth of some tumor cell lines, while other cell types were virtually unaffected in their growth by TGF-β3, eg., human foreskin fibroblasts.

Introduction

Transforming growth factor-β (TGF-β) was initially discovered by its ability to promote the anchorage independent growth of rat NRK fibroblast cells in the presence of TGF-α (Delarco and Todaro, 1978). However, it is now recognized that TGF-β is a multifunctional regulator of cell growth and differentiation (Sporn, *et al.*, 1987; Massague, 1987). For example, TGF-β stimulates the growth of osteoblasts (Centrella, *et al.*, 1987), fibroblasts (Roberts, *et al.*, 1985) and Schwann cells (Ridley, *et al.*, 1989) and inhibits the growth of many epithelial cells (Moses, *et al.*, 1985), endothelial cells (Baird and Durkin, 1986) and T- and B- lymphocytes (Kehrl, *et al.*, 1986a; Kehrl, *et al.*, 1986b). TGF-β blocks adipogenesis (Ignotz and Massague, 1985), myogenesis (Massague, *et al.*, 1986) and hematopoiesis (Keller, *et al.*, 1989) and promotes chondrogenesis (Rosen, *et al.*, 1988) and epithelial cell differentiation (Masui, *et al.*, 1986). TGF-β has multiple physiological roles, including in embryogenesis, tissue-repair, bone remodeling, immunoregulation and inflammation. Potential clinical applications of TGF-β, include wound healing, bone repair and immunosuppression (Sporn, *et al.*, 1987).

TGF-β3 is a member of the structurally and functionally related family of growth factors. Thus far, three homodimeric isoforms have been identified in mammals, termed TGF-β1, TGF-β2 and TGF-β3. TGF-β1 and TGF-β2 have been purified from several tissues, transformed cells and conditioned media of cell lines (Assoian, *et al.*, 1983; Cheifetz, *et al.*, 1987; Seyedin, *et al.*, 1987; Wrann, *et al.*, 1987; Ikeda, *et al.*, 1987). The

cDNA cloning for human TGF-β1 revealed that it is synthesized as a 390 amino acid precursor, with a hydrophobic signal peptide at the N-terminus and the bioactive mature part encoded by the C-terminal 112 amino acid residues (Derynck, *et al.*, 1985). The N-terminal region of the TGF-β1 precursor is glycosylated (Brunner, *et al.*, 1988). Proteolytic processing occurs at the signal sequence and the tetrabasic cleavage site. The human cDNAs for TGF-β2 (de Martin, *et al.*, 1988) and TGF-β3 (ten Dijke, *et al.*, 1988; Derynck, *et al.*, 1988) indicate that they may also be produced as larger secreted precursor proteins, with proteolytic processing sites at 112 amino acids from the C-terminus. The amino acid sequences for the three human TGF-β isoforms are highly homologous in their mature domains (approximately 70-80%), whereas the homology is much reduced in the amino acid sequences of the N-terminal precursor regions (25-35%). Cloning of TGF-βs from other mammalian and avian species indicate that all TGF-β sequences are highly conserved in evolution (Sporn, *et al.*, 1987).

Although TGF-β1 and TGF-β2 have comparable biological activities, striking quantitative differences in biological activities have been reported. TGF-β2, but not TGF-β1, induces mesoderm in *Xenopus* ectodermal explants (Rosa, *et al.*, 1988) and TGF-β1 has been claimed to be a 100 fold more potent growth inhibitor of hematopoietic stem cells for this gene (Ohta, *et al.*, 1987). Presently, little is known about the biological activities of TGF-β3 as the cDNA for this gene was only been recently identified (ten Dijke, *et al.*, 1988; Derynck, *et al.*, 1988).

Recombinant TGF-β1 has been overexpressed in Chinese Hamster Ovary (CHO) cells using dihydrofolate reductase (*dhfr*) gene amplification (Gentry, *et al.*, 1987). Both mature and precursor forms were present in the conditioned media of the transfected cells. Recombinant TGF-β1 is produced in a latent form. Acidification of the conditioned medium was necessary to release the noncovalently bound mature TGF-β1 from the N-terminal precursor region to obtain biological activity (Gentry, *et al.*, 1987).

We report here the eukaryotic expression of human TGF-β3, its purification to apparent homogeneity, and some effects of TGF-β3 on the stimulation or inhibition of cell growth. Like TGF-β1 and -β2, TGF-β3 appears to be a potent growth modulator of multiple cell types.

Materials and Methods

Cell Lines

Stock cultures of cell lines were maintained at 37°C in a humidified 5% CO_2-95% air atmosphere as follows: Mv1Lu mink lung epithelial cells, A549 lung adenocarcinoma, A375 melanoma, A2058 melanoma, WiDr colon adenocarcinoma, MCF7 breast cancer and Huf human foreskin fibroblasts in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone); MG-63 osteosarcoma and MNNG osteogenic sarcoma in minimal essential medium (MEM) supplemented with 10% fetal bovine serum; G292 osteosarcoma in McCoys medium supplemented with 10% fetal bovine serum; K562 chronic myelogenous leukemia, KG-1 acute myelogenous leukemia; Hut 78 T cell lymphoma and U937 histiocytic lymphoma in RPMI supplemented with 10% fetal bovine serum; CHO cells deficient in *dhfr* were propagated in Ham F12 medium supplemented with 10% dialysed fetal bovine serum (Hyclone).

General Nucleic Acid Procedures

Purification of nucleic acids, restriction enzyme digestion, gel electrophoresis, transfer of DNA and RNA to nitrocellulose, labeling, and hybridization were performed essentially according to established procedures as described by Maniatis, *et al.* (1986).

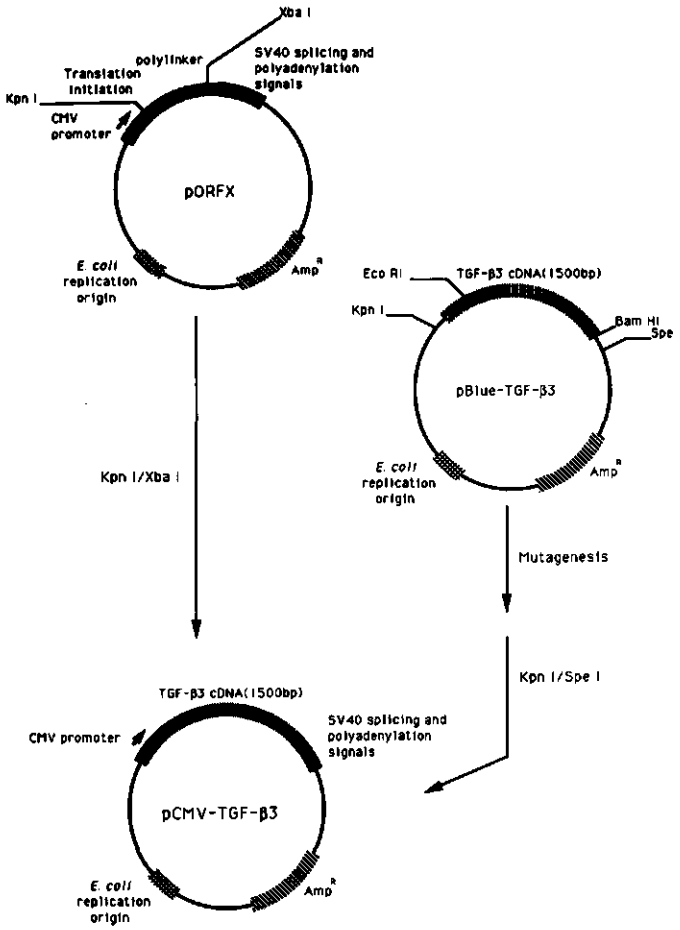

Figure 1: Schematic representation of the construction of the pCMV:TGF-β3 expression plasmid.

DNA sequencing was performed according to Sanger *et al.* (1977). *In vitro* mutagenesis of the flanking sequence, changing the initiation codon CACAC[ATG]A into CCACC[ATG]A, was performed using the method of Nakamaye and Eckstein (1986).

TGF-β3 Expression Construct

A 1500 bp *Alu*1-*Hga*1 restriction fragment of TGF-β3 cDNA, corresponding to nucleotide positions 255 and 1755, respectively (ten Dijke, *et al.*, 1988), which encodes the complete TGF-β3 protein was cloned into the Bluescript plasmid (Stratagene, La Jolla, CA) to yield the plasmid pBlue-TGF-β3. The flanking sequence of the initiation codon was mutagenized to a more efficient translation sequence, according to Kozak (1986), by changing CACAC[ATG]A into CCACC[ATG]A. Mutagenesis was confirmed by DNA sequencing. Subsequently, the mutagenized pBlue-TGF-β3 was cut with *Kpn*I and *Spe*I, two polylinker restriction sites flanking the cDNA insert. This fragment was cloned into the eukaroytic expression vector pORFEX (Bernard, *et al.*, 1987) cut with *Kpn*I and *Xba*I. In this construct (pCMV:TGF-β3) the TGF-β3 cDNA sequence is transcriptionally regulated by the cytomegalovirus immediate early promoter (Figure 1).

DNA Transfection and Gene Amplification

A standard calcium phosphate-DNA precipitation method was used for DNA transfection (Graham and van der Eb, 1973). Both the cytomegalovirus (CMV) promoter-driven TGF-β3 cDNA expression plasmid (5.7 kb) and the plasmid containing the *dhfr* gene driven by its own promoter (2.5kb) were coprecipitated in a ratio of 10 μg to 0.05 μg, respectively, and this calcium phosphate-DNA precipitate was added to 0.5 x 10^6 CHO cells deficient in dihydrofolate reductase (*dhfr*$^-$). Selection of transformants with the *dhfr*$^+$ phenotype was performed in alpha MEM supplemented with 10% dialyzed fetal bovine serum. Colonies that appeared after culturing for 10 to 14 days in selection medium were isolated using cloning cylinders and expanded. For gene amplification, the primary transfectants were selected by increasing concentrations of methotrexate (Mtx) in selective medium. TGF-β3 expression levels of primary transfectants and levels during amplification were measured by RNA cytodot hybridization (White and Bancroft, 1982) normalizing the expression of TGF-β3 mRNA to that of actin.

Biological Assay

The growth of Mv1Lu mink lung epithelial cell is extremely sensitive to TGF-βs (Ranchalis, *et al.*, 1987). These cells were, therefore, utilized to test conditioned media for biological activity of the recombinant TGF-β3 protein. The growth inhibition assay is based on measurement of DNA synthesis as previously described (Iwata, *et al.*, 1985). The cells were subcultured on 96-well tissue culture plates in 100 μl of complete medium at a density that allowed them to be in a growing state throughout the time period of the assay. Conditioned media samples with TGF-β3 were acid activated by treatment with acetic acid (final con. 0.1M) for 30 min followed by neutralization by NaOH/HEPES and assayed in triplicate at several serial dilutions added to each well. The cells were incubated for 72 h at 37°C in a humidified 5% CO_2-95% air atmosphere. At the end of this incubation period, each well was treated for 24 h with 100 μl of complete medium containing 5-[^{125}I] iodo-2-deoxyuridine, [^{125}I]IdUr (1μCi/ml). The monolayers were washed once with phosphate-buffered saline, fixed for 10 min in methanol and air dried for 15 min. The [^{125}I]IdUr incorporated in the cells was solubilized with 200 μl of 1N NaOH and incubated for 20 min at 60°C. The amount of cell growth is measured the amount of [^{125}I]IdUr incorporated into the DNA of actively growing cells. Inhibition of growth was expressed as the percentage of decrease of [^{125}I]IdUr incorporated by cells treated with

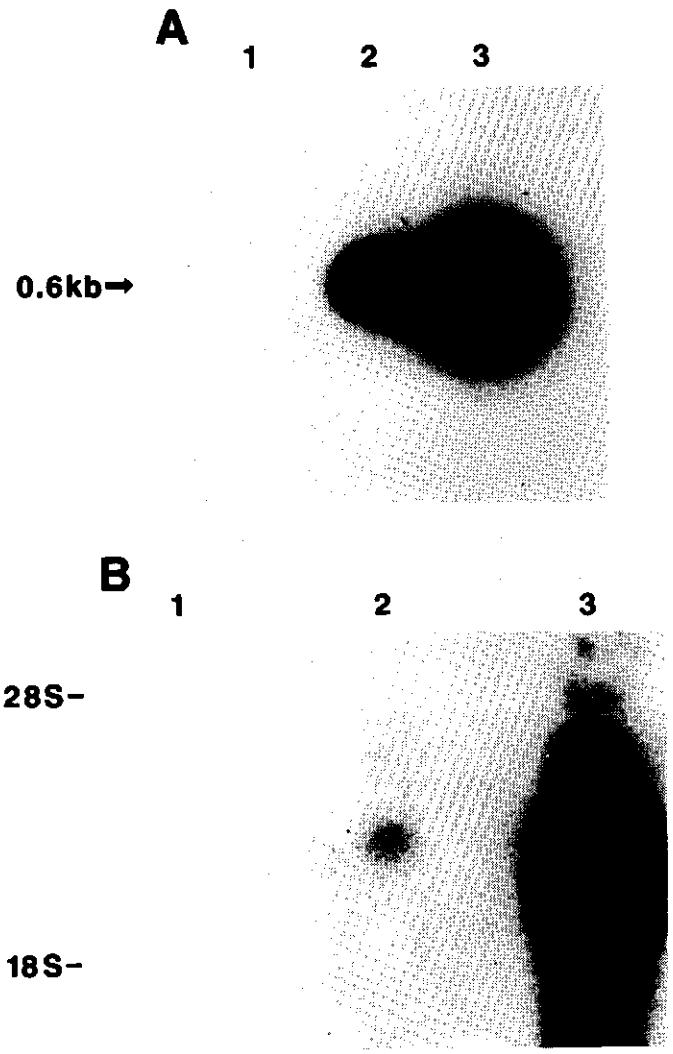

Figure 2: (A) Southern blot of parental CHO cells (lane 1), CHO 6.35 clone (lane 2) and CHO 6.35 with 20 mM Mtx (lane 3). Genomic DNA digested with *Bgl* II (15μg) was fractionated on a 0.8% agarose gel, blotted onto nitrocellulose and probed with 0.6 kb *Bgl* II TGF-β3 cDNA.
(B) Northern blot of parental CHO cells (lane 1), CHO 6.35 clone (lane 2) and CHO 6.35 with 20mM Mtx (lane 3). Total RNA (50μg) was fractionated on a 1.2% agarose formaldehyde gel, blotted onto nitrocellulose and probed with 0.4 kb *Eco*RI-*Sma*I TGF-β3 cDNA. The position of the ribosomal 18S and 28S markers are indicated.

TGF-β3 containing media samples relative to [^{125}I]IdUr incorporated by the untreated control cells.

Peptide Synthesis and Antibody Production

Peptides β3III (DTNYCFRNLEENC) and β3V (YLRSADTTHSTVLGLYNTLNPEAS ASY) corresponding to peptide sequences within the TGF-β3 protein were synthesized on an Applied Biosystems peptide synthesizer (Model 430A) using tert-butyloxycarbonyl (t-Boc) chemistry. The peptides β3III and β3V were coupled to keyhole limpet hemocyanin via glutaraldehyde and used for immunization of rabbits. For specificity studies of the TGF-β3 antisera, the cognate TGF-β1 peptides β1III (DTNYCFSSTEKNC) and β1V (YIWSLDTQYSKVLALYNQHNPGASAAY) and TGF-β2 peptides β2III (AAYCFRNVQDCNC) and β2V (YLWSSDTQHSRVLSLYNTINPEASASY) were also made. Antisera from the immunized rabbits were affinity purified using an affinity matrix composed of the respective peptide β3-antigen coupled to Affi-prep 10 (Bio-Rad, Richmond, CA) (Harlow and Lane, 1989).

Immunodetection

Enzyme-linked immunosorbent assays were used initially to characterize the antibody titers. For Western blotting, proteins were fractionated on 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose (0.1 μm; Schleicher and Schuell, Inc., Keene, NH) in 200 mM Tris, 150 mM glycin, 0.02% wt/vol SDS and 20% vol/vol methanol, using 0.8 mA for 15 h at 4°C (Towbin, *et al.*, 1979). The blots were blocked in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 3% non-fat milk powder for 1 h at room temperature. Subsequently, the blots were incubated with affinity purified antibody (5 μg/ml) in TBST for 1 h at room temperature. After excess of antibody had been washed away with TBST, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA) diluted 1:5,000 in TBST. Color was developed using 5-bromo-4-chloro-3-indolyphosphate and nitroblue tetrazolium (Promega Corporation, Madison, WI) as substrates in AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

TGF-β3 Protein Purification

Conditioned medium was prepared from transfected CHO cells grown to near confluence in alpha MEM supplemented with 10% dialyzed fetal bovine serum in the presence of 20nM Mtx in Nunc cell factories. The cells were washed with phosphate buffered saline (PBS) and incubated with HB CHO serum free medium (HANA Biologics, CA) for 2 hours to eliminate carryover of serum proteins and subsequently incubated with fresh HB CHO serum free medium for 48 hours. A total of six collections (2.5 titers per collection) were performed. The first collection of conditioned HB CHO media contained low levels of TGF-β3 (~1-5ng/ml) and was discarded. Maximum amounts were produced in the 4th through 6th collections (20-50ng/ml). Conditioned media was filtered through a 1 μm glass fiber filter (Micron Separations) and prior to processing, stored in plastic containers at 4°C after addition (with a final concentration) of 0.1 mM PMSF, 4 mM EDTA, 1 mM EGTA, 0.02% sodium azide and 10 mM Tris HCl pH 7.5. The media was concentrated approximately 100 fold using a high capacity, low protein binding Millipore "Pellicon" membrane cartridge (Millipore PLGC Regenerated Cellulose, MW cutoff 10,000). The protein concentration was adjusted to 10 mg/ml and ammonium sulfate (90%, pH 7) was added to 45%, pH adjusted to 7.6 and incubated for 4 hours at 0°C (or overnight). The precipitate was pelleted by centrifugation (10,000 x g for 30 min) and the pellet left to drain for 10 minutes. Subsequently, the pellet was extracted with 50% (vol/vol)

acetonitrile/acetic acid (1M), at 0°C. Twenty five ml of extraction buffer was used per gram of starting protein. The suspension was centrifuged at 10,000 x g for 30 minutes and the supernatant further concentrated using a Minitan concentrator (Millipore). 1M acetic acid was added during concentration to prevent protein precipitation and change the buffer to 1M acetic acid. The concentrated material was chromatographed by Biogel P-60 gel filtration eluting with 1M acetic acid, and peak fractions (as determined by Western blot analysis) concentrated using the Minitan concentrator. Triton was added to a final concentration of 1%. The concentrate was pH adjusted to 7.5 with solid Tris Base (Sigma) and clarified by centrifugation at 10,000 x g for 30 minutes. This material was then chromatographed (at 4°C) on a β3V anti-peptide antibody affinity column (12.5 cm x 0.8 cm), the column washed extensively with 0.1M Tris HCl pH 7.5, 10mM EGTA, 1mM PMSF, 1% Triton X-100, 1M NaCl until no protein could be detected in the eluate. TGF-β3 was then eluted with 50mM glycine (pH 2) into siliconized plastic vials. The TGF-β3 from the antibody affinity column was applied to a Waters C18 reverse phase HPLC column and eluted by a linear gradient of 0-60% acetonitrile 0.1% trifluoro acetic acid (TFA) gradient, a flow rate 0.5ml/minute and monitored at 210nm. Material was aliquoted and stored in the elution solvent at -20°C. Quantitation of chromatographic yields was accomplished by immunoblot analysis using β3III anti-peptide antibody. The yield was about 30-50% of starting material. Purity was assessed by polyacrylamide gel electrophoresis/silver stain analysis.

Amino Acid Analysis and Sequence Determination

Purified TGF-β3 was hydrolyzed with argon-purged, constant boiling 6N HCl containing 1% (vol/vol) phenol at 110°C for 18 hr. Amino acids were derivatized with phenylisothiocyanate and separated with an Applied Biosystems model 130A PTC analyzer. A Waters 840 systems was used for data collection and reduction. Amino acid sequences were determined using an Applied Biosystems model 477A sequencer. Cleaved amino acid derivatives were identified with an on-line Applied Biosystems model 120A PTH analyzer.

Mass Spectrometry

TGF-β3 was analyzed using a Bio Ion 20 (Bio Ion AB, Uppsala, Sweden) time-of-flight mass spectrometer with ionization by ^{252}Cf fission fragments (Sundquist, *et al.*, 1984). A 100 pmol aliquot of TGF-β3 was applied to aluminized Myler, which was previously electro-sprayed with 100 μl of nitrocellulose. The sample disc was spun-dried and washed with 20 μl of 0.1% aquous trifluoroacetic acid (Johnsson, *et al.*, 1986). Spectra were collected for 10 hr at 16000 V accelerating potential. Spectra were calibrated using hydrogen and sodium ions.

NRK-Soft Agar Colony Formation

TGF-β3 was tested for the ability to stimulate normal rat kidney (NRK) fibroblasts to grow in soft agar. A soft agar under layer of 50μl of 0.5% agar (Noble) in 10% fetal bovine serum containing medium was added to a well of 96 well plate. Cells were diluted in 0.5% agar to obtain a final agar concentration of 0.34% agar and 100μl with serial diluted TGF-β3 and 10 ng/ml EGF was added on top of the soft agar underlayer. The cells (with factors) were incubated for 5-7 days at 37°C in a 5% CO_2 atmosphere. At the end of the assay 25 μl 0.05% solution of p-iodonitrotetrazolium violet (INT) was added to the well and incubated another 24 hrs at 37°C. The assay was quantitated on a Biotek 320 plate reader at a wavelength of 540nm.

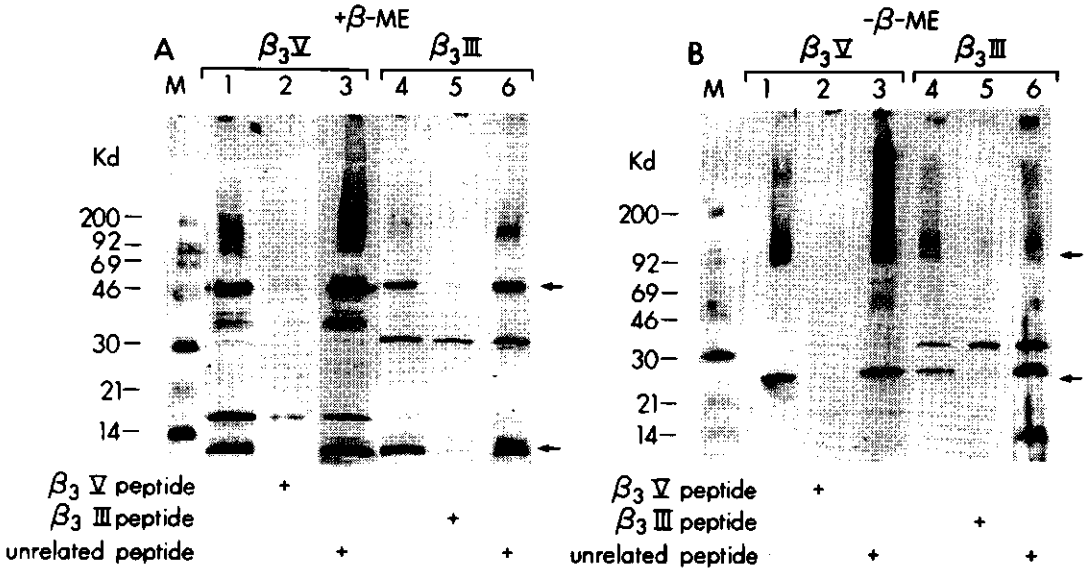

Figure 3: Immunoblot of TGF-β3 produced by CHO transfectant using β3III and β3V peptide antibodies. (A) shows gel under reducing conditions while (B) shows the gel under non-reducing conditions. Lanes 1-3 and 4-6 corresponds to conditioned media immunoblotted with β3V and βVIII antisera, respectively. For peptide blocking experiment the antibody was incubated with 80-fold molar excess of peptide prior to incubation with the blot, lanes 2 and 5 in presence of cognate peptide and lanes 3 and 6 in presence of unrelated peptide. The positions of the mature and precursor TGF-β3 proteins are indicated by arrows.

Growth Regulation of Human Cell Lines

Growth was determined using a modification of the monolayer assay for TGF-β described by Iwata, *et al.* (1985). Non-leukemic cells were subcultured on 96-well tissue culture plates in 100μl of media at a seeding density of $2x10^3$ cells per well. Cells were maintained and assayed in DMEM containing 10% fetal bovine serum and 2% L-glutamine. Aliquots of TGF-β3 of 25ng/ml (~ 1nM) were added to the treated cells. When cells in the untreated control wells were 90% confluent, cells were pulsed 24 hours with 1μCi/ml [^{125}I]IdUr and subsequently harvested.

Leukemic cells (K562, KG-1, Hut 78 and U937) were seeded in 50μl of media. K562 was seeded at a density of $1x10^3$ cells per well in RPMI supplemented with 10% fetal bovine serum. KG-1 was seeded at a density of $3.5x10^3$ cells per well in Iscove's media supplemented with 10% fetal bovine serum. Hut 78 and U937 were seeded at a density of $3.5x10^3$ cells per well in RPMI supplemented with 10% fetal bovine serum. Cell growth was determined by microscopic examination.

Results and Discussion

Recombinant Expression of Human TGF-β3

In order to express recombinant human TGF-β3, we constructed an expression plasmid with the TGF-β3 cDNA under transcriptional control of the early cytomegalovirus promoter (pCMV: TGF-β3, see also Figure 1). The initiation codon of TGF-β3 does not form part of a Kozak consensus sequence (CCACC[ATG]G), which has been shown to influence translation efficiency. In order to promote high yields of the recombinant TGF-β3 protein, the flanking sequence of the initiation codon was mutagenized to a more efficient translation sequence according to Kozak (1986), by changing CACAC[ATG]A into CCACC[ATG]A. Most of the flanking 5' and 3' region of the TGF-β3 encoding sequences were deleted to remove upstream initiation codons that could possibly impede translation efficiency or destabilizing elements that decrease mRNA half-life.

The pCMV: TGF-β3 construct was cotransfected with a *dhfr* marker gene into CHO cells deficient in *dhfr*. A number of clones expressing the *dhfr* positive phenotype were isolated in selective medium. To enhance TGF-β3 expression levels, clones containing higher copy numbers of the TGF-β3 gene were selected by stepwise increasing the concentrations of Mtx. Southern and Northern blots of a primary transfectant and a transfectant selected at 20 nM Mtx, probed with TGF-β3 cDNA specific probes are shown in Figure 2. Both TGF-β3 gene copy number and TGF-β3 expression level increase after Mtx selection.

To determine whether biologically active TGF-β3 protein was produced and secreted by the transfected cells, conditioned media of transfected cells were harvested and tested for growth inhibitory activity against Mv1Lu mink lung epithelial cells. An increase in growth inhibitory activity was observed when cell supernatants before and after Mtx selection were compared (data not shown). The clone CHO 6.35 at 20 nM Mtx secreted about 30 ng/ml in 48 hr, as determined by this bioassay relative to a TGF-β1 standard curve. Acidification of the conditioned media was necessary for detectable levels of biological activity, suggesting that TGF-β3 is made in an inactive latent form, in a similar fashion as found for TGF-β1 expressed in CHO cells (Gentry, *et al.*, 1987).

Immunodetection of TGF-β3

Polyclonal antisera were raised in rabbits against peptides β3III and β3V, which correspond to different regions of the mature TGF-β3 protein. The affinity purified β3III antibody exhibits greater than 300 fold specificity for the β3III peptide compared to the cognate peptide sequences from either the TGF-β1 or TGF-β2. Furthermore, no

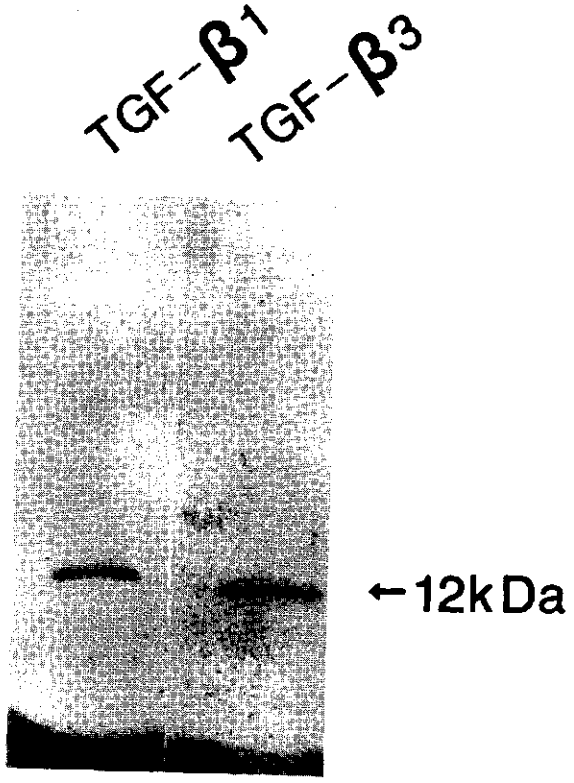

Figure 4: Silver stain of SDS polyacrylamide gel of purified TGF-β3 and TGF-β1 under reducing conditions. The position of 12 kDa marker position is indicated.

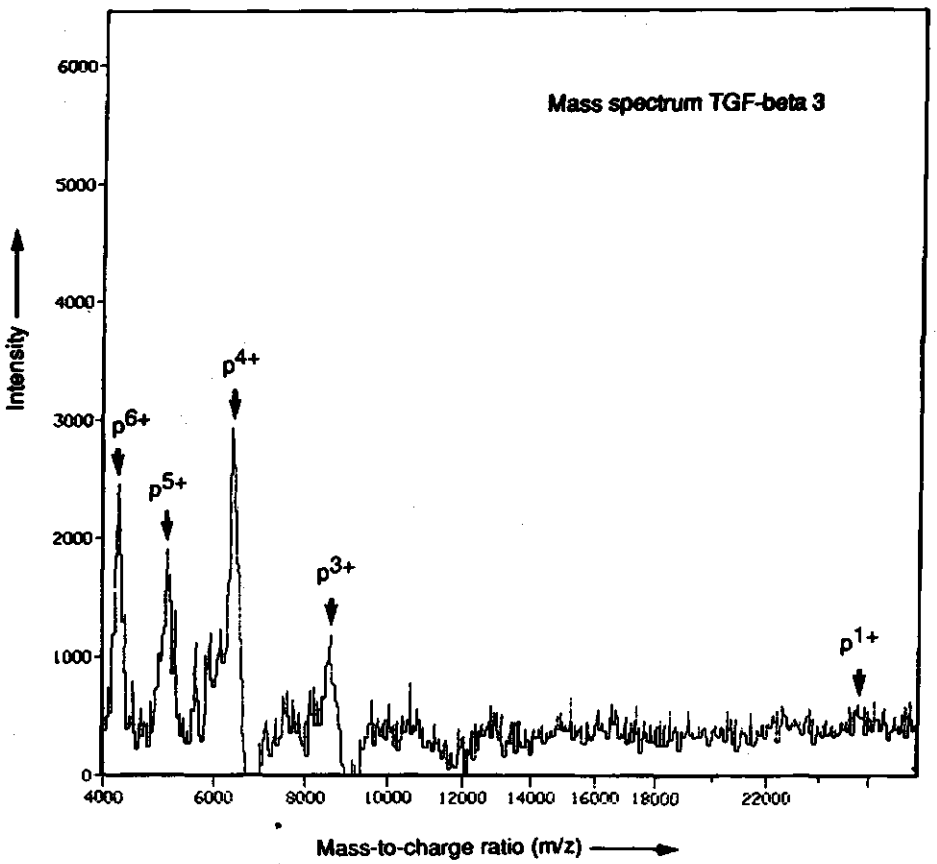

Figure 5: ^{252}Cf plasma desorption time-of-flight mass spectrum of purified TGF-β3. The peaks labeled as p1+, p3+, p4+, p5+ and p6+ refer to the single, triple quadruple, quintuple and sextuple protonated protein ion species, respectively.

significant cross reactivity of this antibody has been observed against either the TGF-β1 or TGF-β2 proteins data (data not shown). The affinity purified β3V antibody exhibits at least a 400 fold selectivity for the β3V peptide compared to the corresponding peptide sequence from TGF-β1. However, whereas this antibody cross reacts with TGF-β2 protein in Western blots, it immunoprecipitates and specifically neutralizes TGF-β3 biological activity on Mv1Lu Mink cells (data not shown).

These antisera were used to identify TGF-β3 in Western blots of conditioned media from transfected cells. By analogy to the proteolytic processing as described by Gentry, *et al.*, (1987), the processed 12 kD mature protein and the uncleaved 50 kD precursor protein were detected under reducing conditions. Under non-reducing conditions, the 24 kD and 100 kD precursor homodimer forms were detected. Specificity of the detected bands was demonstrated by pre-adsorbing the antibodies with the respective peptide immunogen prior to Western blotting (Figure 3).

As expected, based on expression data, the antibody did not detect any TGF-β3 protein in conditioned media of the parental CHO (*dhfr*$^-$) cells (data not shown). Following cleavage of the signal peptide sequence of the precursor form of TGF-β3, one would expect a protein with MW of 43 kDa (under reduced conditions), whereas a 50 kDa protein is detected. Based on the primary sequence of TGF-β3, there are four N-linked glycosylation sites, suggesting the molecular weight differences is likely due to glycosylation. The TGF-β1 precursor fragment expressed in CHO cells was also found to be glycosylated (Brunner, *et al.*, 1988). Within the cells, only the 50 kDa precursor TGF-β was detected by the TGF-β3 peptide antibodies (data not shown).

Purification of TGF-β3

The CHO transfectant 6.35/20 mM Mtx was used as a cell source for the purification of human TGF-β3. Serum free media conditioned for 24 hours using cells grown to near confluence was employed as starting material. This media was filtered through a 1 μm glass fiber filter to remove detached cells and was subsequently concentrated approximately 100 fold using a Pellicon membrane cartridge with 10.000 MW cutoff. Subsequently, the protein in the supernatant was ammonium sulfate (45%) precipitated and the pellet extracted with acetonitrile/acetic acid. This step gave approximately 40-fold purification with no significant loss of TGF-β3 protein. Subsequently, a Biogel P60 gel filtration step was employed. The fractions were analyzed by Western blot analysis and peak fractions of the mature TGF-β3 concentrated. This material was then chromatographed on a β3V anti-peptide antibody affinity column. Finally, TGF-β3 from the antibody column was purified on a reverse phase C18 HPLC column to remove glycine from the antibody affinity column and other contaminants. TGF-β3 eluted at approximately 30% acetonitrile concentration as a single peak. We were usually able to recover 30-50% of the starting material as homogeneous TGF-β3.

The purity and identity of the recombinant TGF-β3 were assessed using several criteria. Analytical gel electrophoresis followed by silver staining showed a single protein species with an apparent mol. wt. of 12 kDa under reducing conditions (Figure 4). When 3 μg of TGF-β3 was loaded onto a polyacrylamide gel, no other proteins were detected, indicating a purity of >95% (the silver stain could detect <50 ng). Comparing TGF-β1 and TGF-β3, shows that recombinant TGF-β3 runs at a lower apparent molecular weight than TGF-β1 under reducing conditions. Possibly this is due to differences in binding of SDS to differences in conformation between the two proteins affecting the migration rate in the polyacrylamide gel. The amino acid composition of purified TGF-β3 corresponded with the composition based upon the predicted amino acid sequence of human TGF-β3. Direct N-terminal sequencing of 30 amino acid residues was in exact agreement with the predicted N-terminus of human TGF-β3 based upon the cDNA sequence, indicating that

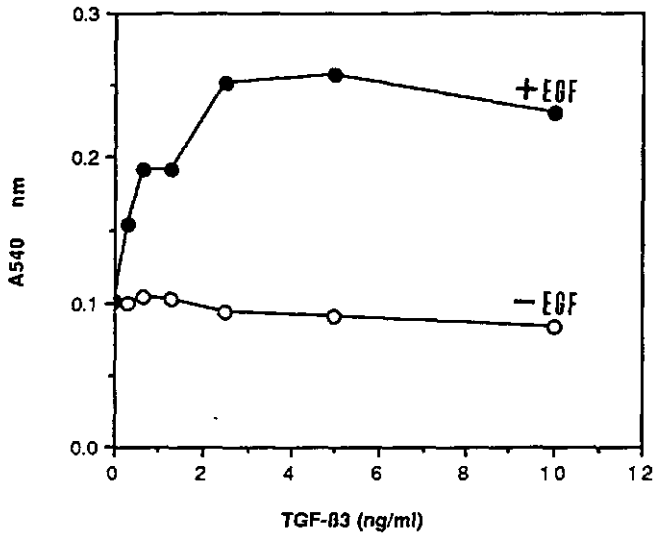

Figure 6: Stimulation of normal NRK rat fibroblast growth in soft agar by TGF-β3. Varying amounts of TGF-β were incubated with NRK cells for 7-10 days. Absorbance in the wells was measured with a Biotek 320 plate reader at a wavelength of 540 nm.

TABLE 1 : Effects of TGF-β3 (1 nM) on Cell Proliferation in Culture

Cell Line	% Inhibition
Human tumor cell lines	
A549 (lung adenocarcinoma)	46
A375 (melanoma)	47
A2058 (melanoma)	88
WiDr (colon adenocarcinoma)	24
MCF 7 (breast carcinoma)	57
G292 (osteosarcoma)	<5
MG-63 (osteosarcoma)	<5
MNNG (osteosarcoma)	<5
K562 (chronic myeloid leukemia)	55
KG-1 (acute myeloid leukemia)	50
Hut 78 (T cell lymphoma)	50
U937 (histiocytic lymphoma)	50
Normal human	
Huf (foreskin fibroblasts)	6

the mature TGF-β3 is proteolytically processed after the multi-basic sequence in an analogous fashion to TGF-β1.

The molecular weight for TGF-β3, as determined with a time-of-flight (TOF) mass analyzer utilizing high energy fission fragment bombardment, was 25430 Dalton, which corresponds with the predicted molecular weight according to the amino acid sequence predicted from the cDNA sequence (Figure 5), this indicates that human TGF-β3 produced in CHO cells is not post-translationally modified

Stimulation of Normal NRK Rat Fibroblasts to Grow in Soft Agar

TGF-β3 was characterized for its ability to stimulate soft agar growth of normal rat fibroblasts (NRK) in soft agar, as is observed for TGF-β1 and TGF-β2 (Ranchalis, *et al.*, 1987). NRK cells were plated with various amounts of TGF-β3 in the presence of EGF. As shown in Figure 6, TGF-β3 is a potent stimulator of NRK soft agar growth (ED_{50} = 1 ng/ml). Although induction of cells to grow in soft agar is characteristic of cell transformation, TGF-β stimulation of extracellular matrix formation is thought to be responsible for the reversible induction of NRK growth in soft agar.

Effects of TGF-β3 on Cells in Monolayer or Suspension

The effect of TGF-β3 on the cell proliferation of a number of cell lines was tested. The cells were treated with 25 ng/ml (~1 nM) of TGF-β3, a concentration which completely inhibits Mv1Lu mink lung epithelial cells. Most of the cell lines were partially inhibited and others (i.e., osteosarcomas) were not affected by TGF-β3. The A 2058 melanoma cell line was the most sensitive human cell line to growth inhibition by TGF-β3. Dose response curves indicated that a plateau response level was reached for each cell line below 1 nM. Comparing the effect of TGF-β3 with TGF-β1 (normalized with respect to protein concentration), showed that both factors have similar qualitative effects, with both factors reaching the same maximum inhibition level (data not shown). Partial inhibition might be explained by the fact that TGF-βs affect the cell cycle. TGF-β1 has been shown to arrest cells in the middle to late G1 phase delaying entry into S phage (Heimark, *et al.*, 1986; Shipley, *et al.*, 1985). Alternatively, the tumor cell lines may be heterogeneous, containing subpopulations that are resistant to growth inhibition by TGF-βs.

TGF-β3, like TGF-β1 and TGF-β2 appears to be a potent growth modulator. However, based upon the activities of TGF-β1 and TGF-β2, TGF-β3 is likely to be a multifunctional growth regulator, not only affecting cell growth but also differentiation and other cell functions. The availability of highly pure recombinant biologically active TGF-β3, as presented in this report, will allow a detailed structural and functional analysis and the evaluation of the multiple clinical applications of TGF-β3.

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CHAPTER SEVEN

Recombinant Transforming Growth Factor Type β3: Biological Activities and Receptor-binding Properties in Isolated Bone Cells

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Recombinant Transforming Growth Factor Type β3: Biological Activities and Receptor-Binding Properties in Isolated Bone Cells

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We have recently cloned the cDNA for transforming growth factor type β3 (TGF-β3), a new member of the TGF-β gene family. We examined the biological effects of recombinant TGF-β3 protein in osteoblast-enriched bone cell cultures. In this report we demonstrate that TGF-β3 is a potent regulator of functions associated with bone formation, i.e., mitogenesis, collagen synthesis, and alkaline phosphatase activity. In a direct comparison between TGF-β3 and TGF-β1, TGF-β3 appeared to be three- to fivefold more potent than TGF-β1. Our cross-linking experiments with iodinated TGF-β showed that in osteoblast-enriched bone cell cultures, both TGF-β3 and TGF-β1 associated with the same three cell surface binding sites. Scatchard analysis of receptor competition studies indicated the presence of high-affinity binding sites for TGF-β3 in the picomolar range. TGF-β3 showed an approximately fourfold-higher apparent affinity than TGF-β1 in overall binding.

Type β transforming growth factors (TGF-βs) are multifunctional growth modulators which play a central role in embryonic development, tissue repair, immunoregulation, fibrosis, and carcinogenesis (18, 26). The two well-described 25-kilodalton (kDa) homodimer forms, TGF-β1 and TGF-β2, share 71% amino acid sequence identity. A heterodimer, composed of one polypeptide chain each from TGF-β1 and TGF-β2, termed TGF-β1.2, has been found in porcine platelets (6). In most assay systems, TGF-β1 and TGF-β2 have similar biological properties, although dramatic differences have also been observed. TGF-β1 has been claimed to be a 100-fold more potent growth inhibitor of hematopoietic stem cells than TGF-β2 (20). In addition, TGF-β2 but not TGF-β1 has been reported to induce mesoderm formation in early frog embryos (23). Differences in tissue distribution and gene regulation also suggest distinct biological roles for various TGF-β isoforms (26).

Recently, we identified another human form of TGF-β, termed TGF-β3. The mature bioactive form of TGF-β3 shares 80% amino acid sequence identity with TGF-β1 and 83% sequence identity with TGF-β2 (28). TGF-β3 mRNA expression in human cells has been found in umbilical cord and in a number of mesenchymal and tumor cell lines (9, 28). We have now expressed recombinant TGF-β3 and purified this protein to homogeneity by using an immunoaffinity column (28a).

TGF-β-like activities are produced by bone cells, and large amounts are found in the extracellular bone matrix, suggesting an important physiological function of TGF-βs in this tissue (4). TGF-β stimulates cell replication and collagen production in cultured fetal rat bone cells (2–5) and induces chondrogenesis of embryonic rat mesenchymal cells (25). In addition, molecules with TGF-β-like activity are released in vitro after bone resorption and may effect a link between the coupled processes of bone formation and resorption during remodeling (22, 27).

The experimental paradigm we have used in this report is to isolate various cell populations from resected bone fragments by sequential collagenase digestions (2–5). The later-released populations are enriched for bone-forming cells with the biochemical characteristics associated with the osteoblast phenotype, such as type I collagen production, elevated alkaline phosphatase activity, and osteocalcin synthesis (17). Studies with such isolated bone cells have shown that TGF-β1 is a potent regulator of cells from the osteoblast lineage (3). On a molar basis, TGF-β1 is one of the most potent mitogens thus far described for osteoblast-enriched cultures from fetal bone. The mitotic response to TGF-β1 is biphasic, with an optimal concentration below 100 pM (2–4). TGF-βs, in addition, alter expression of various activities associated with the osteoblast phenotype: alkaline phosphatase activity is decreased, while the synthesis of type I collagen is enhanced, similar to the effects of TGF-β in a number of other connective tissue systems (2, 3).

TGF-β1 and -β2 appear to act via binding to specific cell membrane receptors linked to as yet unknown intracellular signaling pathways. Virtually all cell types examined to date carry TGF-β receptors, with the exception of retinoblastoma and pheochromocytoma cells (14). The binding of TGF-β1 and TGF-β2 to integral cell membrane receptors has been demonstrated by chemical cross-linking. Three size classes of ligand-receptor complexes have been identified: (i) a 65-kDa glycoprotein; (ii) an 85- to 110-kDa glycoprotein; and (iii) a heterogeneous proteoglycan with an average size of 250 kDa (2, 7, 16, 24, 29). Current evidence suggests that the type I TGF-β receptor is required for TGF-β1's effect on DNA, collagen, and fibronectin synthesis (1, 24).

The present studies were performed to assess the effects of human recombinant TGF-β3 on osteoblast-enriched cultures from fetal rat parietal bone and to characterize the specific binding of TGF-β3 to bone cell surface proteins.

MATERIALS AND METHODS

Test agents. Recombinant human TGF-β3 protein used in these studies was obtained from conditioned medium of Chinese hamster ovary (CHO) cells transfected with an expression plasmid containing the TGF-β3 cDNA. The TGF-β3 protein was purified to homogeneity as judged by

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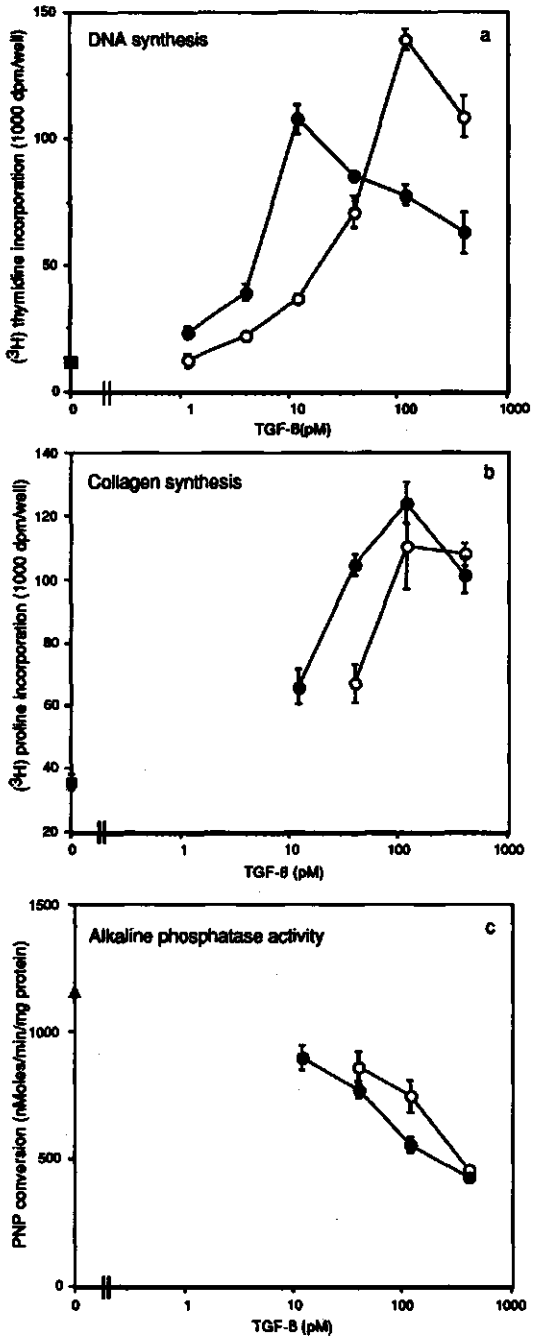
DNA synthesis
a
(3H) thymidine incorporation (1000 dpm/well)
150
100
50
0
0
1
10
100
1000
TGF-ß(pM)
Collagen synthesis
b
(3H) proline incorporation (1000 dpm/well)
140
120
100
80
60
40
20
0
1
10
100
1000
TGF-ß (pM)
Alkaline phosphatase activity
c
PNP conversion (nMoles/min/mg protein)
1500
1000
500
0
0
1
10
100
1000
TGF-ß (pM)

silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28a). Human TGF-β1, purified from platelets, was obtained from Calbiochem (La Jolla, Calif.). TGF-β1 (from Biomedical Technologies, Stoughton, Mass.) and TGF-β3 were iodinated on ice for 15 s by a modification of the method of Hunter and Greenwood (11). The radiolabeled TGF-β was separated from the free iodine by adsorption to a Waters SEP-Pak C18 cartridge, which was pretreated with bovine serum albumin. The iodinated TGF-β was eluted with 80% acetonitrile–0.05% trifluoracetic acid.

Cell cultures. Parietal bones dissected free of adjacent suture lines were obtained from 22-day-old rat fetuses (Sprague-Dawley; Charles River Breeding Laboratories, Wilmington, Mass.) and subjected to five sequential 20-min collagenase digestions as described previously (17, 30). The population of cells released during the first enzyme treatment (population 1) is enriched with less-differentiated fibroblastlike cells, whereas the last three (populations 3 to 5) are enriched with cells expressing features characteristic of the osteoblast phenotype. Cells from population 1 and a pool of cells from populations 3 to 5 were plated at 12,500 cells per cm^2 and cultured in Dulbecco modified Eagle medium as detailed previously (2, 3, 17). After reaching confluence (approximately 6×10^4 cells per cm^2), the cultures were deprived of serum for 20 h; the factors of interest were then added to the cultures in serum-free medium and incubated for an additional 23 h.

DNA synthesis. To examine the mitogenic effect of the test factors, cell cultures were pulse labeled with [^{3}H]thymidine (80 Ci/mmol) for the last 2 h of treatment and lysed by the addition of 0.1% sodium dodecyl sulfate and 0.1 N sodium hydroxide. The insoluble material formed by precipitation with 10% trichloroacetic acid was collected on glass fiber filters, rinsed with ethanol, and measured by scintillation counting. Data are shown as the total amount of acid-precipitable [^{3}H]thymidine incorporated per 0.32-cm^2 culture well.

Protein synthesis. To measure collagen and noncollagen protein synthesis, 2-cm^2 cultures were pulsed with 12.5 μCi of [^{3}H]proline (125 mCi/mmol) per ml for the last 2 h of culture. Cells were rinsed with isotonic buffer (146 mM NaCl, 11 mM dextrose, 35 mM Tris hydrochloride [pH 7.4]) and lysed by freeze-thawing in 0.5% (vol/vol) Triton X-100 (Sigma Chemical Co.). The homogenates were diluted threefold and precipitated with 10% trichloroacetic acid, and the acid-precipitable material was collected by centrifugation. The pellets were acetone extracted, dried, resolubilized in 0.5 M acetic acid, and neutralized with NaOH. The amount of [^{3}H]proline incorporated into collagenase-digestible protein and noncollagen protein was measured as described by Peterkofsky and Diegelmann (21).

Alkaline phosphatase assay. Enzyme activity was measured in extracts prepared from 2-cm^2 cultures following sonication in 0.5% Triton X-100. Hydrolysis of *p*-nitrophenyl phosphate was measured at 410 nm after 30 min (15); data are expressed as nanomoles of *p*-nitrophenol released per minute per milligram of protein.

Receptor assays. Binding of TGF-β1 and TGF-β3 to cell surface-associated binding sites was examined by methods adapted from Massague and Like (16). Confluent cell cultures (4.8 cm^2) were serum deprived for 20 h, rinsed, incubated for 1 h at 37°C, rinsed, and incubated for an additional hour at 4°C in serum-free medium containing 0.4% bovine serum albumin. ^{125}I-labeled TGF-β (80 pM) in the presence or absence of unlabeled TGF-β1 or -β3 was then added for 3 h at 4°C; the cultures were rinsed with cold isotonic buffer and cross-linked by a 15-min incubation at 4°C with 0.5 mM disuccinimidyl suberate. Subsequently, cells were extracted, and the amount of bound ligand was determined in a gamma counter and electrophoresed on 7.5% polyacrylamide gels. Bound [^{125}I]TGF-β was visualized by autoradiography as reported previously (2).

Statistical methods. Data for effects on DNA and collagen synthesis and alkaline phosphatase activity are shown as the means ± standard error of the mean (SEM) and are representative of at least three independent determinations. Binding studies were performed with at least two separate batches of [^{125}I]TGF-β1 or [^{125}I]TGF-β3 and are representative of at least two independent determinations.

RESULTS

Effects of recombinant TGF-β3 on DNA synthesis rates, collagen production, and alkaline phosphatase activity. We examined the effects of recombinant human TGF-β3 and human platelet-derived TGF-β1 in osteoblast-enriched cultures obtained from fetal rat parietal bone (Fig. 1). The protein concentrations of TGF-β3 and TGF-β1 were normalized by using both the colloidal gold assay (Collaborative Research, Bedford, Mass.) and intensity of silver staining on an SDS-polyacrylamide gel. Analogous to our previous reports with native and recombinant TGF-β1 (2, 3), recombinant TGF-β3 had a biphasic stimulatory effect on DNA synthesis, enhanced collagen synthesis, and decreased alkaline phosphatase activity in osteoblast-enriched cultures after 23 h of treatment. TGF-β3 was more potent than TGF-β1, with an approximately three- to fivefold-lower concentration needed for similar half-maximal effects in all three assays.

Relative effects of TGF-β3 on DNA synthesis in osteoblast- and fibroblast-enriched bone cell populations. Earlier studies with isolated bone cells have revealed that TGF-β1 was a potent mitogen for cells within the osteoblast lineage, whereas minimal effects were seen in less differentiated fibroblast-enriched cultures from fetal rat bone (3). In the present studies, we observed that both TGF-β1 (data not shown) and TGF-β3 (Fig. 2) enhanced the rate of DNA synthesis to a greater extent in osteoblast-enriched cultures than in fibroblast-enriched bone cell cultures. In contrast, fetal bovine serum greatly increased the level of DNA synthesis in both cell populations (Fig. 2).

Receptor binding of TGF-β. Cross-linking studies have demonstrated that TGF-β1 associates with three distinct

FIG. 1. Effect of TGF-β3 (●) and TGF-β1 (○) on DNA synthesis (a), collagen synthesis (b), and alkaline phosphatase activity (c). Osteoblast-enriched cultures from fetal rat parietal bone were cultured to confluence and then serum deprived for 20 h prior to a 23-h treatment with either TGF-β3 or TGF-β1 at the concentrations shown. (a) DNA synthesis rates were measured by labeling cells with [^{3}H]thymidine for the last 2 h of culture; acid-insoluble material was assayed by scintillation counting. (b) Collagen synthesis was measured by labeling with [^{3}H]proline for the last 2 h of culture; acid-insoluble cell extracts were digested with nonspecific protease-free bacterial collagenase, and radioactivity was determined in the enzyme-released supernatants. (c) Alkaline phosphatase activity was measured in cell extracts by hydrolysis of *p*-nitrophenyl phosphate to p-nitrophenol (PNP). Data are the means ± SEM of four to six replicate cultures per condition.

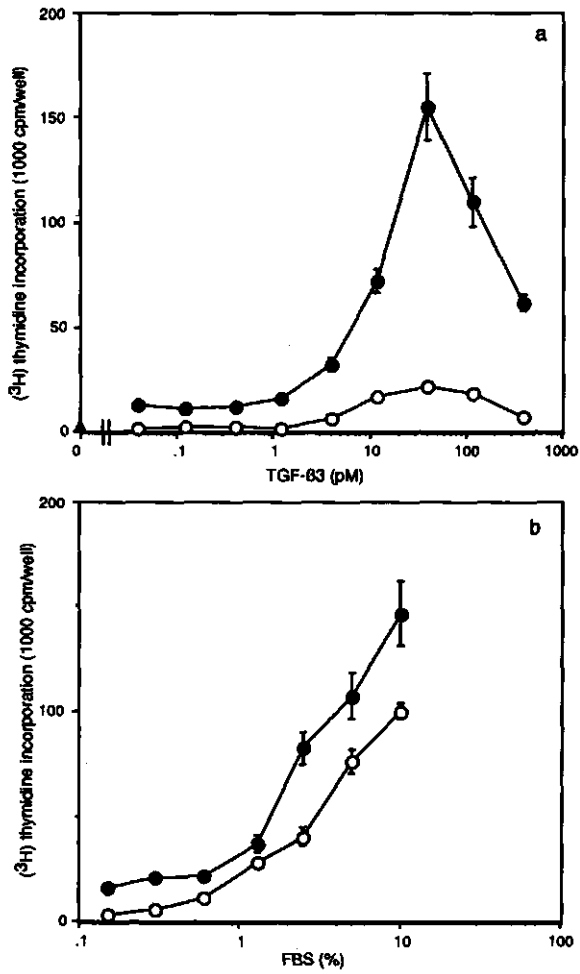

FIG. 2. Effect of TGF-β3 (a) and fetal bovine serum (b) on DNA synthesis of osteoblast-enriched (populations 3 to 5) (●) and fibroblast-enriched (population 1) (○) cells. Confluent cell cultures were serum deprived for 20 h prior to a 23-h treatment with TGF-β3 or fetal bovine serum (FBS) at the concentrations shown. DNA synthesis rates were measured by labeling cells with [^{3}H]thymidine for the last 2 h of culture; acid-insoluble material was assayed by scintillation counting. Data are the means ± SEM of four replicate cultures per condition.

classes of integral cell surface binding sites. We and others have previously reported TGF-β ligand-receptor complex types I, II, and III on fetal rat bone cells that correspond to bands of 65, 85, and >200 kDa when analyzed by polyacrylamide gel electrophoresis (2, 24). The type III receptor is most prominently labeled, followed by the type II and type I receptors, with approximate labeling ratios of 5:2:1, respectively. When [^{125}I]TGF-β1 and [^{125}I]TGF-β3 binding to osteoblast-enriched cultures was compared by these methods, we observed an identical labeling pattern by both ligands, and similar labeling ratios among the three binding sites (Fig. 3). Competition binding studies between [^{125}I] TGF-β1 (80 pM) and 40 to 2,560 pM unlabeled TGF-β1 and TGF-β3 demonstrated that TGF-β3 bound with a three- to fourfold-greater affinity (Fig. 4), and a significant displacement was observed for each receptor class (Fig. 5). The LIGAND program (19) was used to analyze the competition binding data and indicated an overall dissociation constant (K_d) of 200 pM for TGF-β1 and an overall inhibition constant (K_i) of 50 pM for TGF-β3.

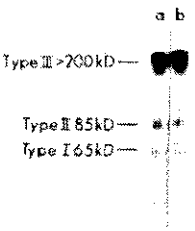

FIG. 3. Cell surface-associated binding of [^{125}I]TGF-β1 and [^{125}I] TGF-β3. A confluent monolayer of osteoblast-enriched cell culture was affinity labeled by incubation in the presence of [^{125}I]TGF-β1 (lane a) or [^{125}I]TGF-β3 (lane b), followed by treatment with disuccinimidyl suberate. Detergent-soluble cell extracts were displayed by gel electrophoresis and autoradiography. The positions of the three labeled TGF-β receptor types are indicated (in kilodaltons).

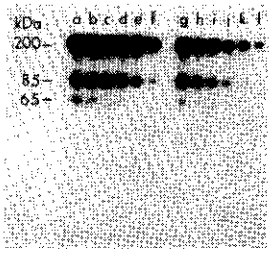

FIG. 5. Relative displacement at cell surface binding sites by TGF-β1 and TGF-β3 in osteoblast-enriched cultures form fetal rat bone. Confluent cell cultures were serum deprived for 20 h, rinsed, and incubated with 80 pM [^{125}I]TGF-β1 in the absence or presence of unlabeled TGF-β1 and TGF-β3 for 3 h at 4°C. The cell lysates were fractionated by electrophoresis on 7.5% polyacrylamide gels, and the bound ligand was visualized by autoradiography. Lane a, No addition; lanes b to f, unlabeled TGF-β1 at 80, 160, 320, 640, and 1,280 pM, respectively; lanes g to l, unlabeled TGF-β3 at 40, 80, 160, 320, 640, and 1,280 pM, respectively.

DISCUSSION

In the present studies we observed that TGF-β3 produces biochemical effects in fetal rat bone-derived cell cultures analogous to those of TGF-β1 (2–4). Like TGF-β1, TGF-β3 enhances DNA and collagen synthesis and decreases alkaline phosphatase activity in osteoblast-enriched cultures from fetal rat bone, but has minimal effects on DNA synthesis in fibroblastlike cells from the same tissue. As in our present and earlier studies with TGF-β1 (2–4), the mitogenic effect of TGF-β was biphasic, with significantly less activity at higher TGF-β concentrations. This may result from complex interactions between signals generated at different binding sites in these cultures. At lower TGF-β levels, the signal generated through the higher-affinity complex would predominate; at higher TGF-β concentrations, the moderating effect of a separate counteracting signal, produced by binding to the lower-affinity site, might then become evident. A more complete understanding of the intracellular signals induced by TGF-β occupancy at each binding site will be required to address this question directly, however.

Receptor-binding studies indicate that in osteoblast-enriched cell cultures, TGF-β3 associates with each of the three binding sites previously demonstrated with TGF-β1 and TGF-β2. The higher affinity of TGF-β3 compared with

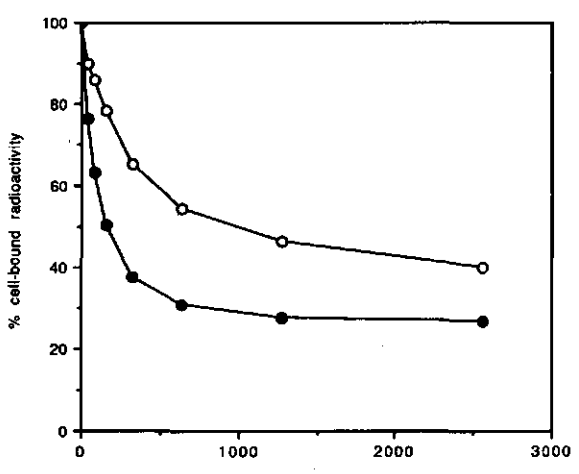

FIG. 4. Competitive binding between TGF-β1 and TGF-β3 in osteoblast-enriched cultures form fetal rat bone. Confluent cell cultures were serum deprived for 20 h, rinsed, and incubated with 80 pM [^{125}I]TGF-β1 in the presence of unlabeled TGF-β1 (○) or unlabeled TGF-β3 (●) for 3 h at 4°C. The cells were lysed, and the amount of bound radioligand was determined in a gamma counter. The maximum amount of [^{125}I] TGF-β1 bound in the absence of unlabeled competitor was 7,100 cpm, or 3.8% of its input concentration. The values shown are the average for duplicate culture wells and are representative of two separate experiments.

TGF-β1 correlates well with the relatively greater biological activity of TGF-β3.

Differences in activity and binding between TGF-β3 and TGF-β1 observed in these experiments could be due to subtle species sequence variations and the heterologous nature of the assay systems, i.e., human TGF-βs have been analyzed with rat cells. This is doubtful, however, since TGF-β3 and TGF-β1 are highly conserved through evolution. For example, the human and chicken mature polypeptide sequences are identical for TGF-β1, and there is only a single conservative amino acid substitution for TGF-β3 (8, 9, 12, 13, 28). Another explanation for these findings could be the sources from which each of these TGF-β preparations were acquired. TGF-β1, purified from human blood platelets, was obtained commercially, and recombinant TGF-β3 was purified to homogeneity from cell culture medium. We believe this second possibility is also unlikely due to the inherent stability of these proteins and because we have observed similar results with multiple preparations of the TGF-βs.

Recently, Graycar et al. (10) found that recombinant TGF-β3 has similar biological activities to TGF-β1 and -β2, but with different relative potencies depending on the cell type. Consistent with our data presented in this report and our observation on TGF-β3 growth inhibition of mink cells (K. K. Iwata, unpublished data), TGF-β3 has been found to be more potent than TGF-β1 in inhibiting DNA synthesis of mink cells and keratinocytes and more potent in stimulating DNA synthesis in AKR-2B cells (10). Receptor competition with [^{125}I]TGF-β1 showed that TGF-β1 competed more effectively than TGF-β3 in AKR-2B cells (10). In contrast, we observed that TGF-β3 competed more effectively than TGF-β1 in osteoblast cultures, an effect more consistent with its relatively greater biochemical potency. These differences could be due to experimental factors such as cell types, cell density, and method of radiolabeling.

Unlike our present results with TGF-β3, analogous studies comparing porcine blood platelet-derived TBF-β1 and TGF-β2 (R and D Systems, Inc., Minneapolis, Minn.) showed no consistently different effects on DNA synthesis or any correlation between mitogenic activity and ligand occupancy at type I TGF-β-binding sites in fetal rat osteoblast-enriched cultures (M. Centrella, unpublished results). This suggests that each of the TGF-βs differentially couples to binding sites and/or differs in the proportion of receptor ligand interaction resulting in postreceptor signals, but this possibility will require a better understanding of the nature of the TGF-β receptor that mediates increased DNA synthesis in these cells.

The precise role and mutual interactions of specific growth regulators in bone metabolism, such as the TGF-βs, fibroblast growth factors, platelet-derived growth factors, insulin-like growth factors, and the bone morphogenetic proteins, have yet to be determined. However, all TGF-β isoforms examined to date are potent mitogens for osteoblast-enriched cultures from fetal rat bone and enhance bone matrix protein synthesis (2–5, 22, 25, 27, 31; this study). Bone grows rapidly during development and early life. In addition, bone is continuously remodeled throughout the life span by cellular events that incorporate bone resorption and subsequent bone formation. Furthermore, in contrast to the healing process that occurs in most other connective tissues, bone repair involves the formation of new bone rather than scar tissue. TGF-β may stimulate precise steps in bone formation during each of the three physiological processes (development, remodeling, and fracture repair). It is possible, therefore, that different members of the TGF-β gene family may have a more important or direct role in each of these processes. It will be of great interest to understand the role of the various TGF-β isoforms in normal and aberrant bone formation, to determine how the different TGF-β isoforms may be used in the treatment of metabolic bone disease, and how to utilize TGF-βs therapeutically to accelerate the process of fracture repair.

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CHAPTER EIGHT

Distinct Transforming Growth Factor-β Receptor Subsets as Determinants of Cellular Responsiveness to Three TGF-β Isoforms

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Distinct Transforming Growth Factor-β (TGF-β) Receptor Subsets as Determinants of Cellular Responsiveness to Three TGF-β Isoforms*

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Characterization of the three mammalian transforming growth factor-β (TGF-β) isoforms, TGF-β1, -β2, and -β3, indicates that TGF-β3 is somewhat more potent (ED_{50} = 0.5 pM *versus* 2 pM) than TGF-β1 and TGF-β2 as a growth inhibitor of the Mv1Lu mink lung epithelial cell line. In the fetal bovine heart endothelial (FBHE) cell line, however, TGF-β1 and -β3 are at least 50-fold more potent than TGF-β2 which is a very weak growth inhibitor (ED_{50} ≥ 0.5 nM). Thus, as growth inhibitors, TGF-β1 and -β3 resemble each other more than TGF-β2. The presence of serum α_2-macroglobulin in the FBHE cell assays decreases the biological potency of TGF-βs, in particular TGF-β2. This effect of α_2-macroglobulin, however, is not sufficient to explain the low responsiveness of FBHE cells to TGF-β2. Evaluation of the role of TGF-β receptors as determinants of cell-specific responsiveness to TGF-β isoforms indicates that TGF-β1, -β2, and -β3 have similar affinity for the membrane proteoglycan, betaglycan. They differ, however, in their ability to bind to receptor types I and II which are implicated in TGF-β signal transduction. TGF-β1 is similar, albeit not identical, to TGF-β3 and much more potent than TGF-β2 as a competitor for binding to the overall population of receptors I and II in all cell lines tested. A subset of receptors I and II has been identified in Mv1Lu cells which has high affinity for TGF-β2 (K_D ~ 10 pM) and binds this factor at concentrations that are biologically active in Mv1Lu cells. This receptor subset could not be detected in FBHE cells, suggesting that cell-specific differences in the level of high affinity TGF-β2 receptors may lead to cell-specific differences in responsiveness to this isoform. Thus, despite their structural and biological similarities, TGF-β1, -β2, and -β3 diverge in their ability to bind to receptors in a manner that correlates with their potency as growth inhibitors.

Transforming growth factor-β (TGF-β)[1] is a term that refers to the dimeric products of various genes, five to date, identified in vertebrates by isolation of the proteins or by cDNA cloning (for reviews see Refs. 1 and 2). TGFs-β are secretory polypeptides that act as paracrine inhibitors of cell proliferation, regulators of cell differentiation, immunosuppressors, and regulators of cell adhesion and extracellular matrix deposition. The three isoforms identified in mammals are TGF-β1, -β2, and -β3. TGF-β1 and -β2 have been purified from various natural sources, and their biological activity and receptor interactions are known with some detail (1, 2). The cDNAs for human and chick TGF-β3 have been isolated (3–5), and the human recombinant protein has been recently purified to homogeneity (6, 7). Expression of these TGF-β isoforms is active throughout embryonic development and into adulthood (8–11). Histochemical localization studies have shown expression of TGF-β1, -β2, and -β3 in discrete regions of many tissues with characteristic temporal and spatial patterns (1, 2). Physiologically, TGFs-β are thought to play a major role in morphogenetic events during embryogenesis, adult tissue recycling, and repair.

The retention of various TGF-β isoforms through evolution is of interest. The extent of identity between the sequences of TGF-β1, -β2, and -β3 is relatively high, ranging from 71% (β1 *versus* β2) to 76% (β2 *versus* β3) (12). Even more remarkable is the level of conservation of the individual TGF-β isoforms. There is >97% identity between the mature TGF-β1 sequences from various mammalian and avian species, and the same is true for TGF-β2 and -β3 (1, 2). Arguably, the high degree of conservation of the mature TGF-β sequences reflects the existence of evolutionary pressure to conserve distinct biological properties in each TGF-β isoform. Indeed, TGF-β1 and -β2 can show marked differences in biological potency in certain cell types (13–17), although their biological potencies are similar in many other cell types (6, 14, 18, 19).

The relationship between receptor binding affinity and biological potency of the various TGF-β isoforms, however, has been unclear. Binding of TGF-β1 and TGF-β2 to most mammalian cells is mediated by two coexisting cell surface glycoproteins of 53 kDa (type I) and 70–85 kDa (type II), respectively, and by the *N*-glycosylated core of betaglycan, a 200–400-kDa cell surface proteoglycan (1). Based on the phenotype of a panel of TGF-β-resistant Mv1Lu cell mutants, it is thought that receptor components I and II are involved in signal transduction since both components are frequently lost in the mutants (20, 21). However, receptor components I and II have much higher affinity for TGF-β1 than for TGF-β2 (13, 14, 18, 22). This marked difference has been paradoxical in cells that respond equally well to TGF-β1 and TGF-β2. By studying the basis for the differential response of cells to TGF-β1, -β2, and -β3, we have identified a previously unknown level of TGF-β receptor heterogeneity that may clarify this paradox.

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; DMEM, Dulbecco's modified Eagle's medium; ^{125}I-dU, ^{125}I-iododeoxyuridine; α_2M, α_2-macroglobulin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FBHE, fetal bovine heart endothelial; PAI-1, plasminogen activator inhibitor-1.

EXPERIMENTAL PROCEDURES

Growth Factors—TGF-β1 and -β2 were purified to homogeneity from bovine bone (23). Porcine TGF-β2 (18) was purchased from R&D Systems. TGF-β3 was purified to homogeneity from serum-free medium conditioned by Chinese hamster ovary cells expressing a transfected human TGF-β3 cDNA, as previously described (7). Homogeneity of the TGF-β preparations was assessed by silver staining of electrophoresis gels overloaded with TGF-β samples. Quantitation of these TGF-β preparations was done by amino acid analysis and by comparison with TGF-β standards on silver-stained electrophoresis gels.

Cell Lines—Stock cultures of cell lines were maintained at 37 °C in a humidified 5%/95% CO_2:air atmosphere as follows: FBHE fetal bovine heart endothelial cells (American Type Culture Collection, CRL 1395) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (GIBCO) and 100 μg/ml endothelial cell mitogen (Biomedical Technologies); Mv1Lu mink lung epithelial cells (American Type Culture Collection) in minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum (GIBCO); BRL-3A Buffalo rat liver cells (American Type Culture Collection) in DMEM supplemented with 10% bovine calf serum; and L_8E_9 rat skeletal muscle myoblasts (24) in DMEM supplemented with 20% fetal bovine serum.

DNA Synthesis (Growth Inhibition) Assays—For growth inhibition assays (25), Mv1Lu cells were seeded in 24- or 48-well multicluster dishes at a density of 30,000 cells/cm² in medium supplemented with 0.2% fetal bovine serum and 10 mM Hepes, or 15,000 cells/cm² in medium supplemented with 10% fetal bovine serum for experiments shown in Fig. 2*B*. After cells had attached (2–4 h), the medium was replaced with fresh medium containing the indicated concentrations of TGF-β. Cultures were incubated for 24 h, the last 2 or 4 h in the presence of 0.5 μCi/ml ^{125}I-deoxyuridine (^{125}I-dU; Du Pont-New England Nuclear). Incorporation of ^{125}I-dU into DNA was measured as previously described (25).

FBHE cells were seeded in 24-well multicluster dishes at a density of 50,000 cells/well in DMEM supplemented with 10% calf serum unless otherwise indicated. 12–15 h later, medium was replaced with 0.5 ml of the same fresh medium containing the indicated concentration of TGF-β. Cultures were incubated for 48 h, the last 18 h in the presence of 1 μCi/ml ^{125}I-deoxyuridine, and incorporation of ^{125}I-dU into DNA was measured.

TGF-β Receptor Assays—Bovine TGF-β1, porcine TGF-β2, and human recombinant TGF-β3 were iodinated by the chloramine-T method as previously described (14). Affinity labeling of confluent cell monolayers with ^{125}I-TGF-β1, -β2, or -β3 and disuccinimidyl suberate was done as previously described (26).

Preparation of α_2-Macroglobulin-depleted Serum—Rabbit anti-human α_2M antibody (4.2 mg) (Sigma) was immobilized by overnight incubation at 4 °C with 0.4 ml of protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) in phosphate-buffered isotonic saline containing calcium and magnesium and then washed with 5 volumes of the same solution. This immobilized anti-α_2M preparation bound up to 10 mg of bovine α_2M/ml of beads. Control beads were prepared with rabbit preimmune serum. Fetal bovine serum (GIBCO) (1 ml of serum/0.3 ml of beads) was incubated with immobilized α_2M antibody overnight at 4 °C to remove endogenous α_2M. Efficiency of depletion was assayed by Western immunoblot using anti-α_2M antibody and ^{125}I-protein A, and known amounts of bovine α_2M (Boehringer Mannheim) as standards.

RESULTS

Growth Inhibitory Activity of TGF-β1, -β2, and -β3—Mv1Lu mink lung epithelial cells and FBHE cells are potently growth inhibited by TGF-β1 but differ in their growth inhibitory response to TGF-β2 (Ref. 17 and Fig. 1). Under normal assay conditions, TGF-β2 is as potent as TGF-β1 in Mv1Lu cells but is much less potent than TGF-β1 in FBHE cells (Fig. 1). We selected these two cell lines to determine the activity of recombinant TGF-β3 as a growth inhibitor using a DNA synthesis assay. TGF-β3 was somewhat more potent (ED_{50} = 0.5 pM) than TGF-β1 and TGF-β2 (ED_{50} = 2 pM) as a DNA synthesis inhibitor in Mv1Lu cells. In FBHE cells, TGF-β3 (ED_{50} = 35 pM) was somewhat less potent than TGF-β1 (ED_{50} = 7 pM) but much more potent than TGF-β2 (Fig. 1*B*). TGF-

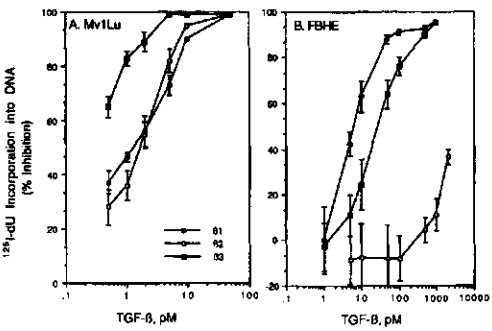

FIG. 1. **Inhibition of ^{125}I-dU incorporation into DNA by TGF-β1, -β2, and -β3.** Sparse cultures of Mv1Lu cells in medium containing 0.2% fetal bovine serum or FBHE cells in medium containing 10% calf serum were incubated for 24 h (Mv1Lu) or 48 h (FBHE) with the indicated concentrations of TGF-β1 (●), TGF-β2 (○), or TGF-β3 (■). Incorporation of ^{125}I-dU into DNA was then measured and is expressed as the percent decrease relative to the value in parallel cultures that did not receive TGF-β. Data are the mean of triplicate values ± S.E.

β2 was a very poor (ED_{50} > 2 nM) inhibitor of DNA synthesis in FBHE cells under the conditions of this assay.

R1B and S1B are mutant Mv1Lu cell clones defective in type I TGF-β receptor binding and TGF-β receptor signaling, respectively (20). R1B and S1B cells, which are resistant to TGF-β1 and β2, were not growth-inhibited by TGF-β3 (data not shown) suggesting that the same receptors and signaling pathways were involved in the growth inhibitory action of these three TGF-β isoforms.

Effect of Serum Factors and Cell-specific Determinants on the Potency of TGF-β Isoforms—Mv1Lu cell growth inhibition assays as those shown in Fig. 1*A* were normally done in the presence of a very low (0.2%) serum concentration whereas the FBHE cell growth inhibition assays were carried out in the presence of 10% bovine calf serum due to cell viability requirements. To search for possible causes of the differential response to TGF-β1, -β2, and -β3 in FBHE cells, we investigated whether bovine serum factors present in the FBHE cell growth inhibition assay could selectively decrease the potency of TGF-β2. When fetal bovine serum was used instead of regular calf serum in the FBHE cell assay, TGF-β1 and -β3 were still 50-fold more potent than TGF-β2 as growth inhibitors; however, a stronger growth inhibitory response to TGF-β2 was observed under these assay conditions (ED_{50} = 400 pM) (Fig. 2*A*) as compared with assays performed in the presence of bovine calf serum (see Fig. 1*B*). Growth inhibition assays were carried out with Mv1Lu cells in the presence of high serum concentrations to determine whether serum factors might also differently affect the potency of various TGF-β isoforms in these cells. In the presence of serum, TGF-β1 was 3-fold more potent than TGF-β2 in Mv1Lu cells (Fig. 2*B*). In no case, however, was the difference in potency between TGF-β1 and TGF-β2 in Mv1Lu cells as marked as in FBHE cells (compare Figs. 1*A* and 2*B* with Figs. 1*B* and 2*A*).

α_2M is an abundant serum component that can bind TGF-β (27–30) and has higher affinity for TGF-β2 than for TGF-β1 (30). Addition of 200 μg/ml exogenous α_2M (the concentration of α_2M that would be provided by the presence of approximately 1% calf serum or 12% fetal bovine serum in

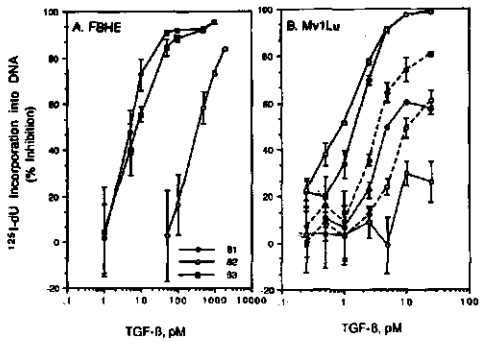

FIG. 2. **Effect of serum on inhibition of ^{125}I-dU incorporation into DNA by TGF-β1, -β2, and -β3.** *A*, sparse cultures of FBHE cells in medium containing 10% fetal bovine serum, or *B*, Mv1Lu cells in medium containing 0.2% bovine calf serum (——), 10% fetal bovine serum (– – –) or 5% fetal bovine serum plus 5% bovine calf serum (· · · ·) were incubated for 48 h (FBHE) or 24 h (Mv1Lu) with the indicated concentrations of TGF-β1 (●) or TGF-β2 (○). Incorporation of ^{125}I-dU into DNA was then measured and is expressed as the percent decrease relative to the value in parallel cultures that did not receive TGF-β. Data are the mean of triplicate values ± S.E.

the assays)[2] markedly decreased the growth inhibitory response of Mv1Lu cells to TGF-β2 but not to TGF-β1 (Table I).

To determine the effect of α_2M on the responsiveness of FBHE cells, the growth inhibitory response to TGF-β1 and -β2 was assayed in the presence of 10% fetal bovine serum that had been depleted of α_2M by treatment with Sepharose-bound anti-α_2M antibody. This treatment depleted 90% or more of the α_2M present in fetal calf serum (data not shown)[2] and yielded a ≤10 μg/ml final concentration of α_2M in the assays. Fetal calf serum that had been treated with Sepharose-bound preimmune rabbit IgG was used as a control. Although depletion of α_2M increased somewhat the growth inhibitory response to TGF-β2, TGF-β2 was still much less potent than TGF-β1 under these assay conditions (Table I and data not shown). Thus, cellular determinants or medium components other than α_2M appeared to be the major cause of the low responsiveness of FBHE cells to TGF-β2 in growth inhibition assays.

High Affinity TGF-β2 Receptor Subset in Mv1Lu Cells—The ability of TGF-β1, -β2, and -β3 to bind to cell surface receptors was compared by performing binding saturation experiments with Mv1Lu cells using radiolabeled derivatives of each TGF-β isoform. Cells were incubated with increasing concentrations of ^{125}I-radiolabeled TGF-β1, -β2, or -β3, followed by the addition of disuccinimidyl suberate to affinity-label and individually examine each TGF-β binding component. Binding to receptor types I and II was detectable with 5 pM concentrations of all three radiolabeled TGF-β isoforms (Figs. 3 and 4*A*). However, a marked difference in receptor saturation patterns emerged as the concentration of radiolabeled ligands was increased. Thus, saturation of the type II receptor component by ^{125}I-TGF-β1 or ^{125}I-TGF-β3 was half-maximal at 25 pM and continued to increase as the concentration of these ligands was increased up to 250 pM. In contrast, binding of ^{125}I-TGF-β2 to this receptor component reached a maximum at 25 pM (Figs. 3 and 4*A*). Furthermore, the maximal labeling intensity of receptor types I and II reached with this ligand was only about 25% of that reached with either ^{125}I-TGF-β1 or ^{125}I-TGF-β3 (Fig. 3).

Similarly, BRL-3A rat epithelial cells subjected to affinity labeling saturation experiments with ^{125}I-TGF-β1 and -β2 also showed a limited (10% of total) subset of receptor types I and II that bound ^{125}I-TGF-β1 and -β2 with high affinity and a larger subset that bound with high affinity only ^{125}I-TGF-β1 (Fig. 4*B*). In contrast to these three cell lines, FBHE cells and L_6E_9 rat skeletal muscle myoblasts failed to provide evidence for the presence of a subset of high affinity TGF-β2 receptors (data not shown).

^{125}I-Labeled TGF-β1, -β2, and -β3 showed only limited differences in their ability to saturate betaglycan in Mv1Lu cells. Binding to betaglycan increased as the concentration of these ligands was increased to 250 pM, with ^{125}I-TGF-β2 displaying a somewhat higher affinity than ^{125}I-TGF-β1 and ^{125}I-TGF-β3 (Fig. 3).

Receptor Competition Patterns of TGF-β1, -β2, and -β3—The next series of experiments examined the ability of native TGF-β1, -β2, and -β3 to compete with tracer radioligands for binding to cell surface receptor components. TGF-β1, β2 and β3 competed effectively against ^{125}I-TGF-β1 for binding to betaglycan in Mv1Lu cells. Half-maximal inhibition of labeling was obtained with concentrations of TGF-β1, -β2, or -β3 in the 100–250 pM range (Fig. 5). However, the three TGF-β isoforms differed in their ability to compete for receptor types

[2] Based on quantitation of α_2M by Western immunoblot (see "Experimental Procedures").

TABLE I

Effect of α_2M on cell responsiveness to TGF-β1 and TGF-β2

Cell line	Fetal bovine serum in the assay	α_2-Macroglobulin	Growth inhibition[a]	
			TGF-β1[b]	TGF-β2[b]
	%	μg/ml	%	
Mv1Lu	0.2	0[c]	86 ± 9	88 ± 4
	0.2	200[c]	94 ± 1	33 ± 10
FBHE	10, untreated	(~170)[d]	90 ± 1	27 ± 10
	10, preimmune IgG-treated[e]	(~170)[d]	85 ± 1	42 ± 8
	10, anti-α2M IgG-treated[e]	(≤10)[d]	87 ± 4	57 ± 7

[a] Growth inhibition refers to percent decrease in ^{125}I-dU incorporation into DNA as compared with control cultures that did not receive TGF-β. Assays were performed in triplicate. The results shown are representative of three separate experiments.

[b] The concentrations of TGF-β1 and TGF-β2 used were 2.5 pM (Mv1Lu cells) or 50 pM (FBHE cells).

[c] Final concentration of exogenously added α_2M.

[d] Estimated final concentration of α_2M contributed by serum present in the assays.

[e] Fetal calf serum was treated with either Sepharose-bound preimmune IgG or Sepharose-bound anti-α_2M IgG. See text for details.

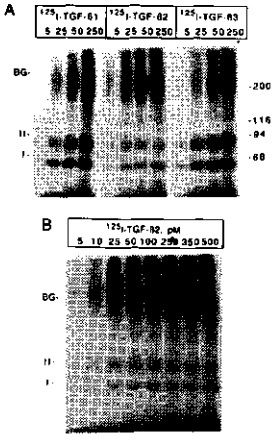

FIG. 3. **Receptor saturation patterns of TGF-β1, -β2, and -β3 in Mv1Lu cells.** *A*, cells were affinity-labeled with the indicated concentrations of ^{125}I-TGF-β1, ^{125}I-TGF-β2, and ^{125}I-TGF-β3. Shown are autoradiograms of polyacrylamide electrophoresis gels containing detergent extracts of the affinity-labeled cells. Autoradiographic exposure of individual panels were adjusted in order to compensate for differences in specific activity of the three ^{125}I-TGF-β preparations used. *B*, a repeat of the experiment with a more detailed range of ^{125}I-TGF-β2 concentrations. The positions of receptor types I, II, and betaglycan (*BG*) as well as the molecular mass in kilodaltons of protein standards run in parallel are indicated.

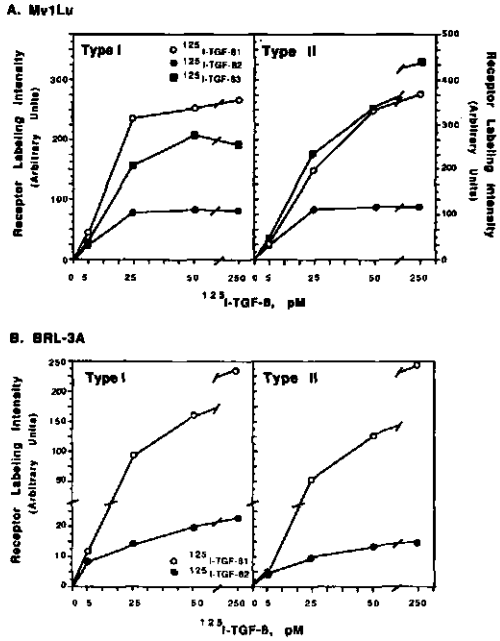

FIG. 4. **TGF-β receptor saturation patterns in Mv1Lu and BRL-3A cells.** *A*, the intensity of the autoradiographic bands corresponding to receptor types I and II in Fig. 3*A* was determined by densitometry. *B*, the intensity of the autoradiographic bands corresponding to receptor types I and II in BRL-3A cells affinity-labeled with the indicated concentrations of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 was determined by densitometry.

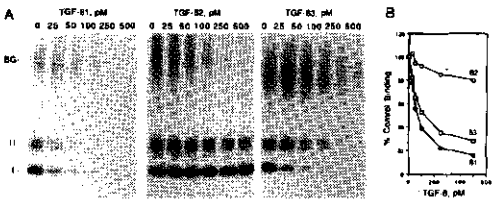

FIG. 5. **Receptor competition patterns of TGF-β1, -β2, and -β3 in Mv1Lu cells.** *A*, cells were affinity-labeled with 25 pM ^{125}I-TGF-β1 in the presence of the indicated concentrations of TGF-β1, -β2, or -β3. Shown are autoradiograms of polyacrylamide electrophoresis gels containing detergent extracts of the affinity-labeled cells. *B*, cell-associated radioactivity after incubation with 25 pM ^{125}I-TGF-β1 in the presence of the indicated concentrations of TGF-β1 (●), -β2 (○), or -β3 (□). Data are expressed as percent relative to the value in control cells incubated with ^{125}I-TGF-β1 alone. The apparent difference in mobility of betaglycan in the *TGF-β3 panel* compared with the *TGF-β1* and *-β2 panels* is due to the fact that the samples were run in separate gels.

I and II. As previously reported (18), labeling of these receptor components by ^{125}I-TGF-β1 was reduced to half or less by the presence of 25 pM TGF-β1 but was only minimally affected by 500 pM TGF-β2 (Fig. 5). The previously untested isoform, TGF-β3, competed for the type I receptor with a potency similar to TGF-β1. However, TGF-β3 competed for the type II receptor with half or less of the potency of TGF-β1 (Fig. 5). Similar TGF-β binding competition patterns were observed with FBHE cells and two additional cell lines, BRL-3A cells (14) which have a TGF-β receptor profile similar to Mv1Lu cells and L_6E_9 cells (31) which have a receptor profile similar to FBHE cells (Fig. 6).

Despite its limited ability to compete with ^{125}I-TGF-β1 or -β3 for receptor types I and II, TGF-β2 competed very effectively for the receptor subset that bound ^{125}I-TGF-β2 in Mv1Lu cells (Fig. 7). TGF-β1 and -β3 were also potent competitors for this receptor subset (data not shown). Taken together, the experiments described in Figs. 3–7 showed that the subset of receptor types I and II that have high affinity for TGF-β2 had properties that made it undetectable when TGF-β2 was used as a competitor of heterologous ligands (TGF-β1 or -β3) in binding assays.

DISCUSSION

Growth Inhibitory Activity of Three TGF-β1 Isoforms—The purpose of this study was, in part, to evaluate the biological properties of the newly isolated recombinant factor, TGF-β3. As it has been recently noted (6) and the results with Mv1Lu cells here confirm, cell lines that respond well to both TGF-β1 and TGF-β2 also respond well to TGF-β3. These responses are to low picomolar concentrations of TGF-β, and if differences in the potency of various TGF-β isoforms exist they are limited to less than 10-fold. Although limited, these differences in potency could be physiologically important.

Vascular endothelial cells that are strongly growth-inhibited by TGF-β1 but not TGF-β2 (Ref. 17 and Fig. 1) provide a more informative system to compare TGF-β isoforms. The results show that TGF-β3 is as potent a growth inhibitor for FBHE cells as is TGF-β1 and is at least 50-fold more potent than TGF-β2. Thus, TGF-β3 is more similar to TGF-β1 than to TGF-β2 as a growth inhibitor in this cell system. Although these three TGF-β isoforms have a similar high degree of

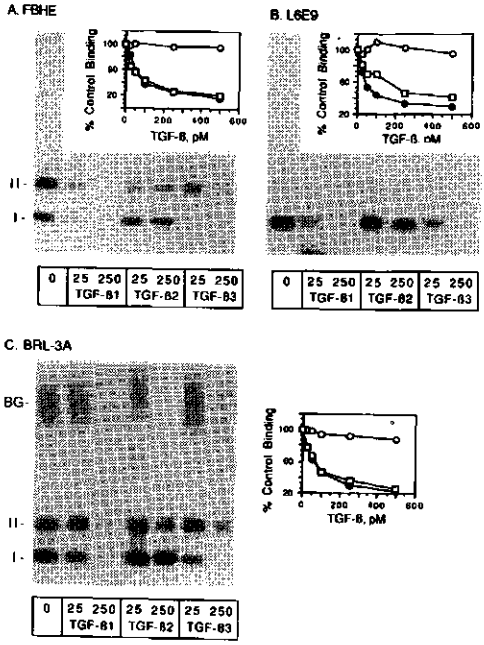

FIG. 6. **Receptor competition patterns of TGF-β1, -β2, and -β3 in various cell lines.** Experiments were carried out as described in Fig. 3, except that the cell lines used were FBHE bovine heart endothelial cells (*A*), L_6E_9 rat myoblasts (*B*), and BRL-3A rat liver cells (*C*). The *inset* in each *panel* depicts cell-associated radioactivity after incubation with 25 pM ^{125}I-TGF-β1 in the presence of the indicated concentrations of TGF-β1 (●), -β2 (○), or -β3 (□). Data are expressed as in Fig. 3.

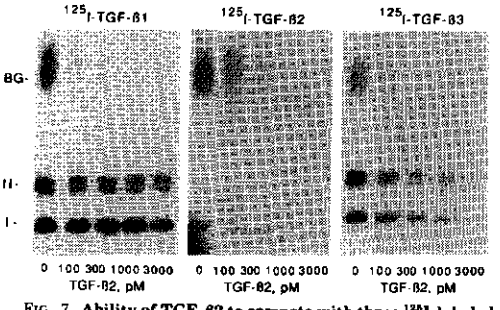

FIG. 7. **Ability of TGF-β2 to compete with three ^{125}I-labeled TGF-β isoforms for receptors on Mv1Lu cells.** Cells were affinity-labeled with 25 pM concentrations of ^{125}I-TGF-β1, ^{125}I-TGF-β2, or ^{125}I-TGF-β3 in the presence of the indicated concentrations of TGF-β2. Shown are autoradiograms of polyacrylamide electrophoresis gels containing detergent extracts of the affinity-labeled cells.

amino acid sequence identity with each other (1–5) some structural feature(s) common to TGF-β1 and TGF-β3, but not TGF-β2, allow these two factors to be potent growth inhibitors for vascular endothelial cells.

α_2M is an abundant serum component that can bind and inactivate TGF-β (27–30). α_2M binds TGF-β2 better than TGF-β1 (30). The results of our experiments based on either adding α_2M to cell cultures or depleting α_2M from serum with immobilized anti-α_2M antibody confirm that α_2M can selectively decrease the responsiveness of Mv1Lu cells to TGF-β2 but fail to demonstrate a major role for this protein as a selective antagonist or inactivator of TGF-β2 in FBHE cell growth inhibition. Many assays in which TGF-β1 and TGF-β2 show similar potency have been carried out in the presence of serum (14, 19, 23). Even in the presence of serum Mv1Lu cells are far more sensitive to growth inhibition by TGF-β2 than FBHE cells are. This indicates that the responsiveness to distinct TGF-β isoforms is strongly influenced by cell-specific determinants.

Subsets of TGF-β Receptors with Distinct Binding Properties as Determinants of Cellular Responsiveness to TGF-β Isoforms—The present results address one additional issue of importance; the results provide evidence for specific subsets of TGF-β receptors I and II that have distinct binding properties. This new evidence relates to previously unresolved questions about receptor interaction with various TGF-β isoforms.

The phenotype of a large panel of TGF-β-resistant Mv1Lu cell mutants implicates receptor types I and II as components of the signal-transducing TGF-β receptor complex (20, 21). The mutants have lost all known responses to TGF-β1, -β2, and -β3. With high frequency, the mutations affect receptors I and II; betaglycan is apparently not affected (20, 21). Furthermore, TGF-β-resistant cell mutants lacking receptors I and II were isolated when either TGF-β1 or TGF-β2 was used in the mutant selection protocol (20, 21). The phenotype of these TGF-β-resistant cell mutants thus implicates receptors I and II as mediators of multiple responses to various TGF-β isoforms. Initially, however, these findings seemed paradoxical because Mv1Lu and other cells respond similarly to various TGF-β isoforms, yet they bind TGF-β2 less avidly than other isoforms. How could TGF-β2 act via these receptors if its ability to bind to them is much lower than that of TGF-β1 or TGF-β3?

A resolution of this issue is suggested in the present study by the detection of a subset of type I and II receptors that has high affinity for TGF-β2. This receptor population can be readily detected in Mv1Lu cells and BRL-3A cells by receptor labeling saturation assays using ^{125}I-TGF-β2 or by receptor labeling competition assays using TGF-β2 to compete with ^{125}I-TGF-β2. The low picomolar concentration range of TGF-β2 (ED_{50} ~ 10 pM) that saturates these high affinity receptors is close to the concentration range (ED_{50} = 2 pM) that induces the biological response of Mv1Lu to this factor. It remains to be determined how the subset of high affinity TGF-β2 receptors is generated. However, both receptor subsets are lost in certain TGF-β resistant Mv1Lu cell mutants (20, 21) suggesting that the high affinity and the low affinity TGF-β2 receptor subsets represent different receptor states rather than separate gene products.

The limited ability of TGF-β2 to compete with ^{125}I-TGF-β1 or ^{125}I-TGF-β3 for the total pool of receptor components I and II and the weak receptor labeling intensity obtained with ^{125}I-TGF-β2 could be explained by the low affinity of TGF-β2 for the larger receptor subset, which is in agreement with previous conclusions (18). The receptors I and II that have high affinity for TGF-β2 could not be properly detected by competition experiments using TGF-β2 against a heterologous ligand (^{125}I-TGF-β1 or ^{125}I-TGF-β3) or by mathematical analysis of equilibrium binding data, given the coexistence of multiple types of TGF-β binding sites on the cell. The role of the larger receptor population that apparently has high affinity for TGF-β1 and TGF-β3 but not TGF-β2 is unclear. We note that the concentration of TGF-β1 and TGF-β3 required

to saturate this pool of "spare" receptors is at least 1 order of magnitude higher than the concentration needed to saturate the biological response in Mv1Lu cells.

Interestingly, we could not detect type I or II receptors with a high affinity for TGF-β2 in FBHE cells which are resistant to growth inhibition by TGF-β2. These marked differences in receptor binding between cell lines may play an important role in the physiology of the TGF-β system. Cell-specific differences in levels of high affinity TGF-β2 receptors might determine cell-specific differences in responsiveness to distinct TGF-β isoforms. Another characteristic of FBHE is their lack of detectable betaglycan, a membrane proteoglycan that binds TGFs-β via its *N*-glycosylated core protein (32–34). Betaglycan can be released by cells and deposited into extracellular matrices (35). Work is in progress to determine if betaglycan selectively affects the responsiveness to TGF-β2 or contributes to the cell's ability to express the receptor subset with high affinity for TGF-β2.

Cell-specific differences in responsiveness to TGF-β isoforms might also be dictated by the nature of each individual response. In this regard, it is important to note that FBHE cells did not discriminate between the three TGF-β isoforms when a relatively short-term effect, up-regulation of plasminogen activator inhibitor-1 (PAI-1), was assayed.[3] In FBHE cells, this response was faster ($t_{\frac{1}{2}}$ = 2–4) than the growth inhibitory response, which required exposure to TGF-β for at least 24 h. Evidence from TGF-β receptor-defective cell mutants suggests that growth inhibition and PAI-1 induction are mediated by the same type of receptors (20, 21). It is possible, then, that a very low level of high affinity TGF-β2 receptors exist in FBHE cells which might be sufficient to mediate the PAI-1 response but be too low to be detectable and to mediate the growth inhibitory response.

In conclusion, the present results indicate that despite certain structural and functional similarities, TGF-β isoforms 1, 2, and 3 can diverge significantly in their potency as growth inhibitors *in vitro*, their recognition by receptors, and their susceptibility to antagonism or inactivation by extracellular factors. The biological differences between TGF-β1, -β2, and -β3 are likely to be physiologically important as suggested by the strict conservation of the individual primary sequences of these factors in vertebrates.

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CHAPTER NINE

Mesoderm Induction in Early *Xenopus* Embryos by Transforming Growth Factor-β3

Peter ten Dijke

Mesoderm Induction in Early *Xenopus* Embryos by Transforming Growth Factor-β3.

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Abstract

The differentiation of mesoderm in *Xenopus* appears to depend upon inductive interactions from the vegetal endoderm upon the overlying ectoderm. Members of the TGF-β and FGF family can mimic and possibly act *in vivo* as mesoderm inducers. Human TGF-β3 was tested and its potency compared with TGF-β1 and TGF-β2 for mesoderm inducing activity on explants of *Xenopus* blastula ectoderm. TGF-β3 was active in this assay, but less potent than TGF-β2. As shown previously, TGF-β1 acting alone had no effect upon mesoderm induction. When TGF-β3 was assayed for induction of muscle formation, it was found to synergize with basic FGF and to be more potent than TGF-β1 in this respect.

Introduction

TGF-βs form a family of related secreted 25 kDa peptides, which affect proliferation and differentiation of many cell types (Roberts and Sporn, 1990). To date, five vertebrate homodimeric isoforms of TGF-β have been described by cDNA cloning or protein purification (Kondaiah, *et al.*, 1990). Three human homodimeric isoforms have been identified, termed TGF-β1 (Derynck, *et al.*, 1985), TGF-β2 (de Martin, *et al.*, 1987) and TGF-β3 (ten Dijke, *et al.*, 1988; Derynck, *et al.*, 1988). Activities for TGF-β1 and TGF-β2 have been characterized in numerous assays. Not much is known about the biological activity of TGF-β3, since it has only recently become available for study. Recombinant TGF-β3 was expressed in Chinese Hamster Ovary (CHO) cells and purified to apparent homogeneity (Graycar, *et al.*, 1989; ten Dijke, *et al.*, 1990a). The three TGF-βs appear to have similar biological activities, but different potencies depending on the cell type and assay used (Jennings, *et al.*, 1989; Ohta, *et al.*, 1987; Tsunawaki, *et al.*, 1988; Graycar, *et al.*, 1989; ten Dijke, *et al.*, 1990b). All three TGF-βs associate with the same three cell surface binding sites (ten Dijke, *et al.*, 1990b).

TGF-βs are highly conserved between mammals and amphibians. The interspecies sequence conservation is higher (>95%) than the intraspecies conservation (Kondaiah, *et al.*, 1990), suggesting an evolutionary pressure to conserve these differences, possibly for functional reasons. Differences in gene regulation resulting in characteristic temporal and spacial expression patterns (Roberts and Sporn, 1990) also suggest distinct biological roles for various TGF-β isoforms.

Immunohistochemistry (Heine, *et al.*, 1987) and *in situ* hybridization (Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988; Pelton, *et al.*, 1989; Akhurst, *et al.*, 1990; Fitzpatrick, *et al.*, 1990) clearly implicate TGF-β's in development. Moreover, TGF-βs are part of a superfamily that include inhibins and activins, mullerian inhibiting substance, bone morphogenetic proteins, the *Drosophila Decapentaplegic* gene product (*DPP-C*), *Xenopus Vg-1* gene product and related murine *VgR-1* gene product, which all play important regulatory roles in development (Roberts and Sporn, 1990).

In amphibian embryogenesis mesoderm is formed and patterned from cells of the animal region by signals originating in the vegetal region (Nieuwkoop, 1969). Mammalian TGF-β2 alone has been reported to induce mesoderm in isolated *Xenopus* animal cap explants (Rosa, *et al.*, 1988) whereas mammalian TGF-β1 can only enhance the mesoderm inducing activity of fibroblast growth factor (FGF)(Kimelman and Kirschner, 1987). Recently, XTC/MIF, a potent mesoderm inducing factor isolated from the *Xenopus* XTC cell line was shown to be homologous to the mammalian activin A (Smith, *et al.*, 1990; van den Eijden-Van Raaij, *et al.*, 1990). The TGF-β family of growth factors induce a variety of dorsal mesodermal cell types including muscle, mesenchyme and mesothelium and notochord, the most dorsal mesodermal tissue (Smith, *et al.*, 1988; Rosa, *et al.*, 1988). The FGF family of growth factors, including acidic FGF, basic FGF, embryonal carcinoma-derived growth factor, and protein products of basic FGF and *int*-2 oncogenes, are capable of inducing ventral type mesoderm differentiation in animal pole explants. In FGF induced explants notochord is rarely observed (Slack, *et al.*, 1987; Kimelman and Kirschner, 1987; Paterno, *et al.*, 1989; Slack, *et al.*, 1989).

The present comparative studies were performed to assess the mesoderm inducing potential of mammalian TGF-β isoforms, in particular TGF-β3, using *Xenopus* animal cap explants and isolated cells.

Materials and Methods

Growth Factors

Human TGF-β1 and porcine TGF-β2 were purchased from R&D Systems, Inc., Minneapolis, MN. TGF-β3 was purified to apparent homogeneity from serum-free medium conditioned by Chinese hamster ovary cells expressing a transfected human TGF-β3 cDNA, as previously described (ten Dijke, *et al.*, 1990a). *Xenopus* basic FGF was prepared by bacterial expression followed by conventional and HPLC heparin columns.

Embryos

Female *Xenopus laevis* were induced to lay eggs by injection of 500 i.u. human chorionic gonadotrophin in 0.5ml phosphate-buffered water (Chorulon, Intervet) into the dorsal lymph sac. The eggs subsequently obtained were fertilized using macerated *Xenopus* testes. Following rotation, eggs were dejellied using 2.5% wt/vol cysteine hydrochloride (adjusted to pH 7.8-8.1 with NaOH). Eggs were then washed thoroughly and allowed to develop in Petri dishes coated with 1.5% wt/vol Noble agar (Difco) in 1/20 normal amphibian medium (NAM) salts (Slack, 1984) with 2 mM-sodium phosphate pH 7.5.

Embryo operations

Operations were carried out on stage-8 embryos in NAM (Slack, 1984) or half-strength NAM containing 5 mM-sodium phosphate pH 7.5 (referred to here as NAM/2). Hair loops and electrolytically sharpened tungsten needles were used to cut out discs of tissue from the center of the animal pigmented hemisphere.

Mesoderm induction assays and histology

Explants were transferred to wells of Terasolin plates (Sterilin) containing 2μl of type 1 agarose (Sigma) and 10-15μl of sample in NAM/2, containing 1mg/ml bovine serum albumin (BSA) in 2-fold serial dilutions of the factors. Explants were maintained

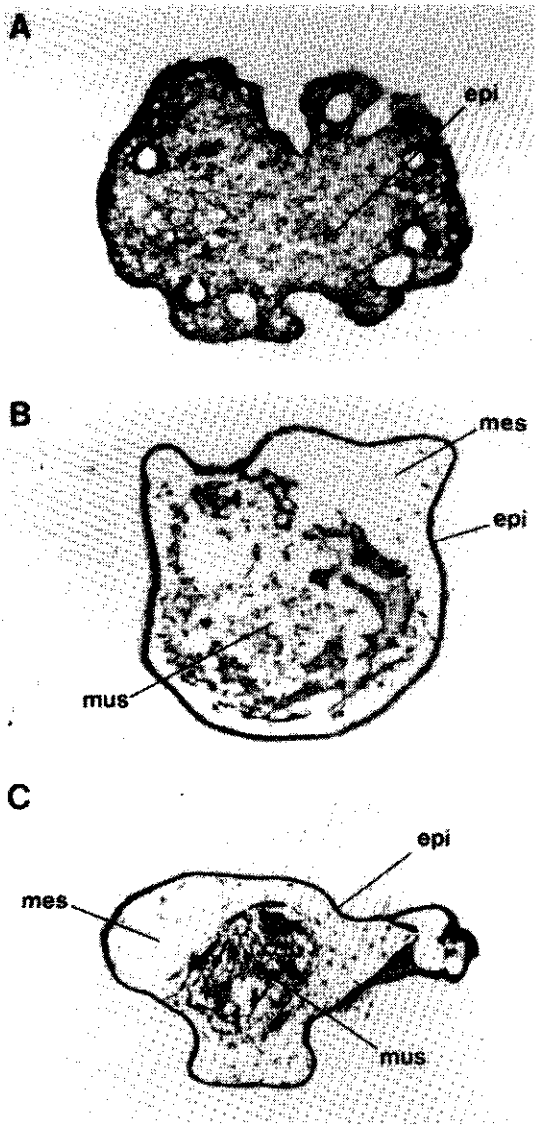

Figure 1

Effect of TGF-β3 on the development of *Xenopus* ectodermal explants. (A) section through control explants after 3 days of culture, which consists entirely of a solid mass of epidermis (epi). (B) and (C) sections through TGF-β3 treated explants after 3 days of culture, showing formation of loose mesenchyme (mes) and muscle (mus).

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CHAPTER TEN

Growth Factors for Wound Healing

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REVIEW/

GROWTH FACTORS FOR WOUND HEALING

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The process of wound healing begins immediately following surface lesions or when skin proteins become exposed to radiation, chemical damage or extreme temperatures. Wound repair requires close control of degradative and regenerative processes, involving numerous cell types and complex interactions between multiple biochemical cascades. Growth factors released in the traumatized area promote cell migration into the wound area (chemotaxis), stimulate the growth of epithelial cells and fibroblasts (mitogenesis), initiate the formulation of new blood vessels (angiogenesis), and stimulate matrix formation and remodeling of the affected region. Animal studies have shown that exogenously added growth factors can accelerate the normal healing process. Growth factors have also been used successfully in humans to treat previously incurable wounds. The most intensively studied growth factors are EGF, FGFs, PDGF, TGF-α, and TGF-βs. Each of these factors is currently the focus of intense commercial development.

Most growth factors were initially named after the biological activity that lead to their isolation. Later, several of these factors were found to be multifunctional, not only affecting cell growth but differentiation, embryogenesis, inflammation, tissue repair, and immune response. Hence, potential therapeutic applications of growth factors include such diverse areas as bone repair, cancer and immunomodulation[1]. In this review, however, we have focused specifically on their role as potent pharmacological agents in soft tissue repair.

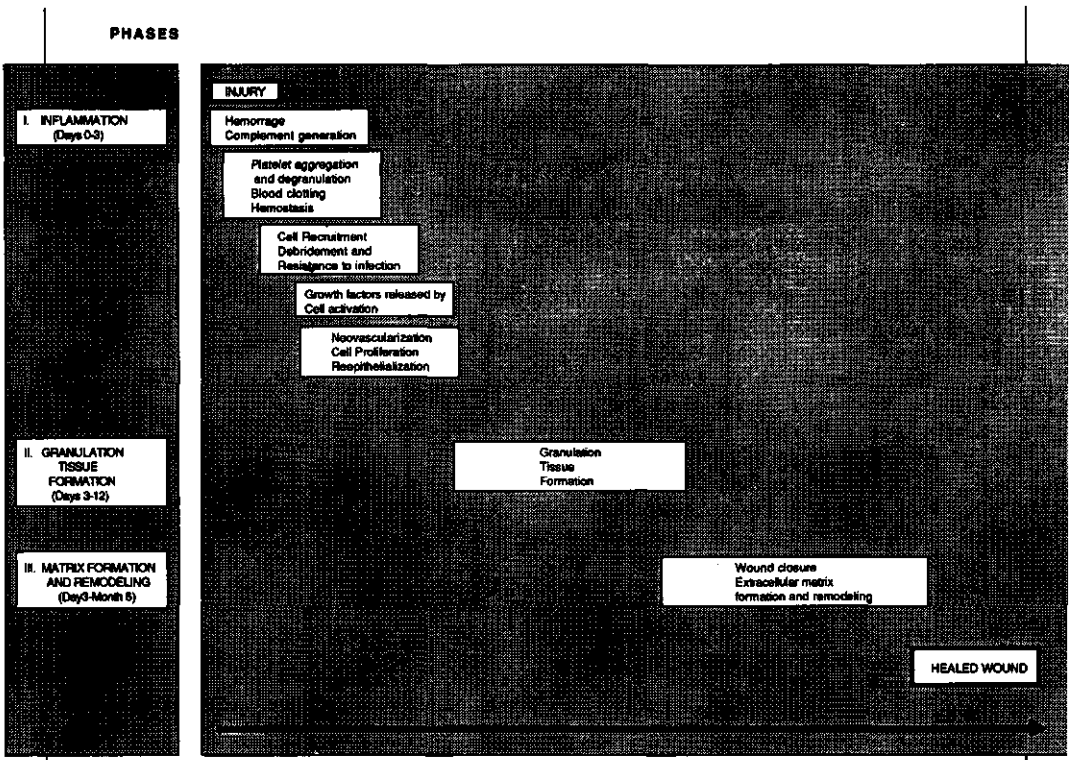
BIOCHEMISTRY OF GROWTH FACTORS

Epidermal growth factor (EGF). EGF is synthesized as a larger precursor protein (Mr=128 kDa), which is proteolytically processed into the mature form of 53 amino acid residues (M_r=5.7 kDa)[2]. EGF is a member of a family of structurally related proteins including TGF-α (see below), amphiregulin and poxvirus growth factors (Table 1)[3]. Human EGF was first isolated from urine and named urogastrone by its ability to inhibit gastric acid secretion[4]. Murine EGF was isolated from salivary glands and found to stimulate precocious eyelid opening and tooth eruption in newborn mice[5] and to be mitogenic for a large number of cell types, including epithelial, fibroblastic and endothelial cells[6]. EGF has also been shown to be chemotactic for epithelial cells[7]. The receptor for EGF is a transmembrane protein (M_r=170 kD) with tyrosine kinase activity[8].

Transforming growth factor-α (TGF-α). TGF-α is also synthesized as a transmembrane precursor (160 amino acid), which is proteolytically processed into a peptide of 50 amino acid residues (M_r=5.7 kDa)[9]. Both TGF-α and EGF bind to the same receptor and share similar biological activities[10]. TGF-α was originally characterized by its ability to act synergistically with transforming growth factor-β (see below) to induce anchorage independent growth of normal rat kidney (NRK) fibroblasts in soft agar and was therefore thought to be involved in malignant transformation[11]. The presence of TGF-α in a variety of human tumors and in cell lines transformed by chemical carcinogens and viral oncogenes supported this initial hypothesis. However, the role of TGF-α in human neoplasia is unclear, since TGF-α expression is also found in activated macrophages and in other normal tissues.

Platelet-derived growth factor (PDGF). PDGF is a heat-stable protein (M_r=30 kDa) that was first purified from blood platelets[12]. Treatment of PDGF from platelets with reducing agents results in the loss of biological activity[13] and yields two polypeptide chains: the A-chain (124 amino acid residues) and the B-chain (140 amino acid residues). The PDGF B-chain corresponds to the proto-oncogene v-*sis*, the transforming gene of simian sarcoma virus[14], suggesting an autocrine role for PDGF-like proteins in neoplasia. PDGF is a potent mitogen for cells of mesenchymal origin (e.g. fibroblasts and smooth muscle cells), but has no effect on the growth of epithelial or endothelial cells[15], which lack PDGF receptors. At low concentrations, PDGF is chemotactic for fibroblasts[16] and has been shown to be both chemotactic and activating for monocytes and neutrophils[17,18]. As in the case of the EGF family, the receptors for PDGF possess intrinsic protein-tyrosine kinase activity[19].

Transforming growth factor-β (TGF-β). In their mature form, TGF-βs are acid- and heat-stable disulfide-linked homodimeric proteins (M_r=25 kDa), with each homodimer consisting of 112 amino acid residues[20]. Two homodimeric forms of TGF-β have been identified (TGF-β1 and -β2), which share significant (>70%) amino acid sequence homology[21,22]. Recently, we reported a new form of TGF-β, designated TGF-β3, which is currently being developed at Oncogene Science for wound healing and other clinical indications[23]. TGF-βs are released in biologically inactive latent forms. Most cells have specific



high affinity receptors for TGF-β1 and β2[20]. Whether TGF-β3 binds to the same or distinct receptors remains to be determined. TGF-βs were characterized originally by their ability to act synergistically with EGF or TGF-α to induce anchorage independent growth of NRK cells[11]. TGF-βs, however, have been found to produce multiple biological effects including mitogenesis, growth inhibition, chemotaxis and induction or inhibition of differentiation, depending upon the cell or tissue type, and the presence or absence of other growth factors[20]. Although the TGF-βs share several biological features, it is becoming apparent that the different forms of TGF-β also possess distinct biological activities and targets[24]. Most of the published work on TGF-βs and wound healing refers to TGF-β1. Thus, unless specifically indicated, the use of the term TGF-β in this review refers to TGF-β1. Other mammalian members of the TGF-β family[20] (see Table 1), include two forms of inhibin and three forms of activin, gonadal proteins that regulate pituitary secretion of follicle stimulating hormone; Mullerian inhibiting substance (MIS), which causes the regression of the female Mullerian duct as part of the development of the male embryo; and bone morphogenic proteins (BMP), a group of polypeptides involved in the induction of cartilage and bone formation[25].

Fibroblast growth factor (FGF). FGFs are members of a family of single-chain proteins of 14-18 kDa, which bind tightly to heparin. Two well defined forms of FGF are acidic FGF (isolated from brain and retina)[26,27] and basic FGF (isolated from brain and pituitary)[28]. Acidic FGF (aFGF) is a nonglycosylated protein of 140 amino acids

FIGURE 1 Soft tissue repair pathways.

(M_r=15.6 kDa) with a pI of 5.6-6.0[29]. Basic FGF (bFGF) is a nonglycosylated protein of 146 amino acids (M_r=16.4 kDa) with a pI of 9.6[30]. In most systems examined, bFGF is more stable and exhibits a 10-fold greater potency than aFGF[31]. Both forms of FGF bind to the same receptor. FGFs are mitogenic for cells of mesodermal origin, such as fibroblasts, vascular endothelial cells, vascular smooth muscle cells, myoblasts, keratinocytes, chondrocytes and osteoblasts[31]. Other members of the FGF family include the products of the *int*-2 and *hst* proto-oncogenes[32,33].

THE WOUND HEALING PROCESS

The process of wound healing can be divided into three main overlapping stages: inflammation, formation of granulation tissue, and matrix formation and remodeling. A schematic representation of these stages is shown in Figure 1.

Inflammation. Tissue injury results in the release of blood components into the wound site. Contact of the blood plasma with tissue protein and the basement membrane activates the clotting cascade. The clot traps plasma protein and blood cells into a fibrin gel thereby inducing hemostasis and providing a matrix for the influx of inflammatory cells. Thrombin, formed during the clotting cascade, stimulates release of alpha granules from aggregated platelets. These alpha granules contain locally acting growth factors such as TGF-β, PDGF and FGF. The combination, concentration and timing of growth factor

release and activation at the site of injury regulate the complex process of wound healing by controlling cell proliferation, chemotaxis and activation of a variety of cellular responses.

Platelet aggregation and blood clot formation cease as the stimuli for initiation dissipate. This cessation involves (i) the generation of protein C (which degrades coagulation factors V and VII[34]), (ii) the release of plasminogen activator (which initiates clot lysis[35]), and (iii) the production of prostaglandins from smooth muscle cells (which inhibit platelet aggregation and PDGF production, thereby providing a feedback control mechanism). The production of growth inhibitors at various steps in the wound healing process may therefore be essential for restraining cell growth in the presence of mitogenic factors.

Neutrophils, attracted by a variety of chemotactic factors, function mainly to remove contaminating bacteria from the injury site. However, they are not essential to wound healing as neutropenia does not interfere with the healing process[36]. Macrophages, like neutrophiles, remove pathogenic organisms and tissue debris, including effete neutrophils. The influx of monocytes and their conversion to macrophages is critical for the initiation of tissue repair. Both cell types are most numerous in the wound site between 3 and 5 days following injury. Macrophages release a variety of biologically active substances, including further amounts of TGF-β and PDGF, which facilitate the recruitment of additional inflammatory cells, augment macrophage-mediated tissue debridement and initiate granulation tissue formation (see below). TGF-β has been shown to be three orders of magnitude more potent than PDGF as a chemoattractant for both monocytes and fibroblasts[37,38]. TGF-β can also stimulate endothelial cell PDGF production. In addition, TGF-β, PDGF and TGF-α have been reported to stimulate their own production, thus providing a way to amplify and sustain their respective physiological effects[39–41].

After an injury site has been sterilized during inflammation, the secreted products of the activated macrophages may become damaging to healthy cells and tissue. At this stage of wound healing, the suppression of macrophage activities may be critical for protecting the surrounding healthy tissue, and for the transition between inflammation and granulation tissue formation. In this regard, therefore, it is interesting to note that TGF-βs have also been reported to deactivate superoxide production from macrophages[42].

Granulation tissue formation. Granulation tissue consists of a dense population of fibroblasts, macrophages and neovasculature embedded in a loose matrix of collagen, fibronectin and hyaluronic acid. TGF-β has a major role in granulation tissue formation. It increases the expression of genes associated with extracellular matrix formation (ECM), such as fibronectin, the fibronectin receptor, various types of collagen and protease inhibitors. TGF-β has been shown to enhance the contraction of a collagen matrix by fibroblasts[43], suggesting its possible involvement in connective tissue contraction. In response to growth factors such as PDGF and TGF-β, the fibroblasts proliferate, migrate into the wound site, and then undergo a series of phenotypic changes to become myofibroblasts, which have characteristics similar to smooth muscle cells. The myofibroblasts align themselves along the radial axis of the newly deposited extracellular matrix within the wound. The myofibroblasts then form interactions with other cells and the extracellular matrix to generate a contractive force that aids in wound closure.

Endothelial cells in the wound proliferate and form new blood vessels (angiogenesis) to supply the injured site with nutrients and oxygen. This nutrient supply is essential for synthesis, deposition and organization of the extracellular matrix. Basic FGF is a potent mitogen for vascular endothelial cells, derived from either large vessels or capillaries[44]. FGF also stimulates endothelial cells to produce a urokinase type plasminogen activator, a protease implicated in neovascularization. In addition, bFGF regulates the synthesis and deposition of various extracellular matrix components[45]. Heparin sulfate, a structural component of the ECM, may function to stabilize FGFs since it protects both bFGF and aFGF from acid or heat inactivation and potentiates their mitogenic activity[46]. It has been suggested that when FGFs are released they become an integral part of the ECM[45]. The hydrolysis of ECM by heparitinase, an inducible enzyme produced by activated platelets and macrophages[47,48], could subsequently lead to solubilization of biologically active heparin sulfate-FGF complexes. Although TGF-βs are not mitogenic for endothelial cells, they are potent angiogenic agents *in vivo*. This is probably due to their ability to chemoattract macrophages and to stimulate macrophages to secrete angiogenic pep-

TABLE 1 Growth factor families.

FAMILY	MEMBERS
Epidermal Growth Factor (EGF)	EGF
	Transforming growth factor-α (TGF-α)
	Vaccinia growth factor (VGF)
	Shope fibroma growth factor (SFGF)
	Myxoma growth factor (MGF)
	Amphiregulin (AR)
Platelet-derived growth factor (PDGF)	PDGF-AA
	PDGF-AB
	PDGF-BB
Transforming growth factor-β (TGF-β)	TGF-β1
	TGF-β2
	TGF-β3
	Inhibins
	Activins
	Mullerian inhibiting substance (MIS)
	Bone morphogenic proteins (BMPs)
Fibroblast growth factor (FGF)	Acidic FGF
	Basic FGF
	hst gene product
	int-2 gene product
Insulin-like growth factor (IGF)	IGF-I
	IGF-II
	Insulin
	Relaxin

TABLE 2 Potential therapeutic applications of growth factors in wound healing.

CATEGORY	SPECIFIC APPLICATIONS
Burns	Thermal burns
	Chemical burns
Surgery	Surgical incisions
	Dental surgery
	Eye surgery
Ulcers	Decubital (bed sores)
	Venous stasis
	Diabetic
	Peptic
Other Indications	Skin grafts
	Bone fractures
	Osteoporosis
	Psoriasis
	Veterinary uses

tides[49] and to their direct affect on capillary formation[50].

Re-epithelialization. Within hours of injury, re-epithelialization begins to restore the integrity of the damaged surface. Re-epithelialization begins with the migration of epithelial cells from the free edges of the tissue across the wound. Within 24 hours, epithelial cells at the original edge of the wound begin proliferating, thereby generating more cells for migration. EGF and other members of the EGF family are chemotactic and mitogenic for epithelial cells[6,7]. Once re-epithelialization is complete, the epithelial cells revert to their non-migrating phenotype and become attached to the basement membrane through hemidesmosomes.

Matrix formation and remodeling. The third phase of wound healing is the gradual dissolution of granulation tissue with devascularization and the loss of cells, fibronectin and type III collagen. The granulation tissue is replaced with connective tissue consisting of a framework of collagen and elastin fibers providing tissue strength and elastic properties, respectively. This framework then becomes saturated with proteoglycans and glycoproteins. Remodeling involves the synthesis of new collagen and the degradation of old collagen. The production of collagen and proteinase inhibitors is stimulated by TGF-β[20], while the production of collagenases by fibroblasts is stimulated by PDGF[51]. The final outcome of matrix formation and remodeling is the scar tissue.

ANIMAL MODELS AND CLINICAL STUDIES

Wound healing involves complex interactions, processes, and numerous cell types, which cannot be completely duplicated *in vitro*. Excluding infection or other complications, the normal process of wound healing often results in complete restoration of tissue function. However, exogenously added growth factors can dramatically accelerate the healing process *in vivo*, suggesting that they are normally present in rate limiting concentrations. More importantly, situations exist in which wound healing is impaired and fails to respond to conventional therapy. These include chemotherapy, venous stasis and diabetic ulcers (Table 2).

Subcutaneous implants. Several animal models have been developed to gauge the efficacy of growth factors in wound healing. A porous subcutaneous chamber is often used as an *in vivo* model for the healing of deep wounds. Chambers of stainless steel wire mesh or polyvinyl alcohol sponges are inserted into the backs of rats. The animal encapsulates the chamber with connective tissue, defining a dead space in which growth factors can be injected. The chambers are removed after one to two weeks and the infiltrated granulation tissue is examined by biochemical and histological techniques. While PDGF, FGF, EGF, and TGF-β have all been demonstrated to stimulate granulation tissue formation using this model system, TGF-β appears to be the most effective[52–54]. To examine the intrinsic role of growth factors in wound healing, the presence of endogenous growth factors in wound chamber models has also been determined. Macrophages isolated from wound chambers were found to express elevated mRNA levels for TGF-α, TGF-β, PDGF, and IGF-1[55]. The concentration of TGF-β was found to peak during the phase of wound healing associated with maximal fibroblast proliferation and collagen synthesis[54].

Surface wounds and burns. Epidermal lesions can be classified as either partial or full thickness wounds. Partial thickness wounds extend to the dermis and heal by epidermal regeneration, using the wound edges as starting points for regrowth. Full thickness wounds extend into the subcutaneous tissue and heal predominantly by granulation tissue formation, re-epithelialization and wound contraction. Pig skin is often used as a model due to its similarities with human skin. Growth factors are most often applied topically in a cream or ointment and healing is measured by the extent of epidermal regeneration and histological evaluation. Experiments using pig skin have shown that repeated doses of EGF accelerated the rate of healing two-fold[56].

Incisional model. Full thickness linear incisions in rat skin is analogous to surgical wounds in humans, involving both epidermal and dermal healing. A single application of a collagen suspension containing either TGF-β or PDGF to the site of incision in rat skin, increased the healing rate[57,58].

Impaired healing model. The efficacy of any wound healing factor is more easily demonstrated in animals with impaired wound healing. Defective wound repair can be induced by chemotherapy, steroids, or streptozotocin. When the effects of EGF, PDGF, TGF-β and insulin on wound healing were compared in adriamycin treated rats, TGF-β was found to be the most effective growth factor[59]. PDGF and EGF alone had no effect. A combination of EGF, PDGF and TGF-β resulted in an almost complete reversal of adriamycin inhibition of wound healing. EGF was shown to prevent methyl prednisolone induced inhibition of wound healing in a wound chamber model[60]. PDGF restored wound healing in streptozotocin-induced diabetic rats, whereas insulin had no effect. A combination of PDGF and insulin, however, stimulated a more rapid increase in collagen deposition than PDGF alone[61]. These animal models indicate that the repair process in the various forms of defective healing requires different growth factors and combinations of growth factors for optimal healing.

Angiogenesis models. Growth factor stimulation of new blood vessel formation *in vivo* can be determined in assays using the avascular rabbit cornea, the hamster cheek pouch and the chick chorioallantoic membrane. All three assays are scored by blood vessel growth toward the angiogenic factor but are difficult to quantitate. Concordant with the *in vitro* observations, FGF's are potent angiogenic factors in all three assays[45]. As discussed previously, TGF-β is a strong growth inhibitor of capillary endothelial cells *in vitro*, but a potent angiogenic factor *in vivo*[49]. Thus, extrapolations between *in vitro* and *in vivo* can not always be made.

Clinical studies. EGF, the most studied growth factor, is currently in clinical trials and under development for a number of indications such as topical administration for wound and burn healing, in opthalamology to accelerate healing of corneal surface abrasions and corneal transplants after cataract removal, and in the treatment of gastric ulcers. Preliminary human clinical data shows that the mixture of growth factors in platelets can restore healing in patients with chronic nonhealing ulcers due to diabetes, chronic venous stasis, systemic lupus erythematosus or rheumatoid arthritis[62,63]. This demonstrates the therapeutic potential of combinations of growth factors for *in vivo* wound healing. Apart from synergism, multiple growth factors will probably be necessary for the complete repair of a wound involving multiple tissues.

Delivery methods and formulation. As the efficacy of growth factors for wound healing indications is established, delivery methods and formulation criteria will become critical issues for the commercial development of the growth factor. Bandages, gels and lotions are currently being used for topical applications of growth factors to the area of soft tissue wounds. For ophthalmic applications, eye drops could be used to deliver growth factors.

The sustained release of growth factors may be more effective than separate applications. Although EGF in

saline solution was unsuccessful in certain wound healing models, EGF in slow release formulations, such as multilamellar liposomes or collagen gels, has been shown to enhance the re-epithelialization and tensile strength of wounds[64]. Moreover, collagen gels are biodegradable and may also enhance the stability of the growth factor. Biodegradable film made of collagen or fibronectin could function as a delivery vehicle, provide protection and act as a substrate for epithelial cell migration and adhesion. As previously mentioned, the presence of specific growth factors at defined times is required for the optimal healing of wounds. An ideal vehicle, therefore, would release growth factors at different rates so that the wound would receive the optimal stimulating and inhibitory signals at the proper time over the entire course of the healing process.

CONCLUDING REMARKS

Growth factors are essential throughout the initial events following injury and throughout later stages of wound repair affecting matrix remodeling and increasing the tensile strength of the scar. Simple topical application of these factors has been shown to increase the rate of wound healing in several model systems and combinations of factors appear to work synergistically. The controlled release of growth factors into the injury site will probably be necessary to prevent overstimulation and excessive scaring. Crude platelet extracts, containing multiple factors, have been shown to have significant therapeutic effects on patients with refractory wound healing. The exact contributions of each factor and the interactions between different factors are currently areas of intensive academic and industrial research. Understanding the normal healing process is essential in order to optimize intervention in the repair process by the addition of exogenous growth factor formulations. Growth factor research may also lead to the development of novel molecules which can inhibit the abnormal growth of cancer cells. A more detailed knowledge of growth factor receptor structure and function, regulation of growth factor genes, and activation of latent forms, may lead to the second generation of wound healing agents.

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CHAPTER ELEVEN

Concluding Remarks and Prospects

CONCLUDING REMARKS AND PROSPECTS

Growth Factors are multifunctional

The aim of the work described in this thesis was to identify novel human growth inhibitory proteins that specifically inhibit the growth and/or cause terminal differentiation of tumor cells. This research resulted in the isolation and characterization of human TGF-β3 (Chapter 4). TGF-β3 was shown to inhibit the growth of several (but not all) types of tumor cells (Chapter 6). Additionally, TGF-β3 was found to stimulate cell proliferation of osteoblasts (Chapter 7) and rat kidney fibroblasts in soft agar (Chapter 6). TGF-β3 was also shown to have an effect on the differentiation on certain target cells, as it induced mesoderm formation in *Xenopus* ectoderm explants (Chapter 9). These results indicate that the effect of TGF-β3 is not strictly growth inhibitory but multifunctional. The particular effect of TGF-β3 on a cell appears to be determined by the cell type, the physiological state of the cell, the presence or absence of other growth factors and binding proteins, and the surrounding microenvironment (eg. extracellular matrix and type of adjacent cells). In general growth factors appear to have pleiotropic effects (Roberts and Sporn, 1988). These multifunctional characteristics clearly implicate the necessity of a tight control of growth factor activities *in vivo*. This requirement may have driven the need to evolve multiple members of a gene family, with different expression and activation patterns, the levels of which are rapidly regulated *in vivo* in response to a variety of control signals.

Growth Factor Families

As the amino acid sequences of more growth factors are elucidated, it is apparent that a number of factors are structurally related and can be grouped into families (Chapter 2). TGF-βs are a family of growth factors of which five different genes, to date, have been identified in vertebrates. Thus far, three homodimeric isoforms, TGF-β1, TGF-β2 and TGF-β3 have been described in mammals. Each of the mammalian TGF-βs is produced as a larger precursor protein which is proteolytically processed into a 112 amino acid mature form. The biologically active mature forms of the TGF-βs share between 70-80% sequence identity, with a strict conservation of all nine cysteines (Chapter 4). A comparison of TGF-β1 and TGF-β3 at the gene level reveals a conservation of all intron-exon junctions with the exception of the first intron, suggesting that the two genes are the result of an ancestral gene amplification (Derynck, *et al.*, 1988).

The interspecies amino acid sequences within the mature domain of individual TGF-β family members is virtually complete (nearly 100%) conservation. The intraspecies divergence between the different TGF-β family members is much higher (approximately 70-80%). On this basis, it is reasonable to assume that each family member within an organism *in vivo* may be functionally distinct. Indeed, significant differences in both biological potency and receptor affinities between TGF-β isoforms on certain cell types have been observed *in vitro* (Chapters 7, 8 and 9; Ohta, *et al.*, 1987; Cheifetz, *et al.*, 1987; Rosa, *et al.*, 1988; Jennings, *et al.*, 1988). However, thus far no unique receptor forms that are isoform specific have been identified.

Different physiological roles for the multiple isoforms of TGF-β is also suggested by differential transcriptional regulation, with each isoform being controlled by unique gene regulatory promoter/enhancer elements. Recently, in a collaboration with Rosemary Akhurst and colleagues (University of Glasgow, UK), we have shown by *in situ* hybridization on tissue sections the expression of TGF-β1, TGF-β2 and TGF-β3 in discrete regions of many tissues with characteristic temporal and spatial expression patterns (Gatherer, *et al.*,

1990). Similar differential expression patterns were found in human and murine embryogenesis, suggesting an evolutionary pressure on preserving the gene regulatory elements of the individual TGF-βs and possible functional difference between different TGF-βs *in vivo*.

Differences in post-transcriptional regulation may represent yet another mechanism for distinct regulation of TGF-βs leading to different *in vivo* activities. The tetrabasic proteolytic cleavage site for TGF-β1 is different from TGF-β2 and TGF-β3. Recombinant expression of TGF-β1 and TGF-β2 in CHO cells indicated that the TGF-β1 precursor is more efficiently processed than the TGF-β2 precursor (Madisen *et al.*, 1990). The precursor domains of the TGF-βs show only 27% sequence identity. These domains may confer biological specificity in tissue targeting. Furthermore, as TGF-βs are produced in an inactive form in which the N-terminal precursor remnant (also termed latency associated peptide) is complexed with the mature protein, the physiological activation mechanism may be specific for the different latent TGF-β complexes. The activity of TGF-βs can also be modulated by TGF-β binding or inactivating proteins. TGF-βs bind to the soluble form of betaglycan (Massague *et al.*, 1990) and to the proteoglycan decorin (Yamaguchi *et al.*, 1990). In addition, in serum, a complex between α2-macroglobulin and mature TGF-β can be isolated, which is inactive (O'Conner-McCourt and Wakefield, 1987; Huang *et al.*, 1988). It has been proposed that α2-macroglobulin may function as an *in vivo* scavenger providing a clearence mechanism to restrict the action range of TGF-β. Comparing the affinities of α2-macroglobulin for TGF-βs show that TGF-β2 binds with a higher affinity than TGF-β1 (Danielpour and Sporn, 1990).

The structural and regulational differences between the TGF-βs are likely to result in different physiological roles. It will be important to address this more directly by (i) immunohistochemical studies with, for example, isoform specific antibodies that distinguish between active and latent TGF-β, (ii) pharmacological studies with the latent/mature TGF-βs, (iii) cloning of the TGF-β receptors, allowing studies for a better understanding of TGF-β signaling and *in vivo* receptor localization, and (iv) experimental manipulation of *in vivo* expression and action of TGF-βs using transgenic animals.

Embryogenesis, Wound Healing and Cancer

There is growing evidence that many of the events which occur in embryogenesis are recapitulated in wound healing and cancer. Processes like mitogenesis, chemotaxis, angiogenesis and formation and remodeling of connective tissue that occur during embryogenesis involve mechanisms similar to those in the processes of wound healing and cancer. The products of proto-oncogenes and growth factors, including TGF-βs (discussed below), play a critical role in these processes as signaling mediators (Chapters 2 and 3). It has been proposed that one of the key differences between physiological processes like embryogenesis and wound healing, versus pathological processes like tumorgenesis, is the activity of proto-oncogene products or growth factors being expressed out of context, ie. at the wrong time or in the wrong cell type. Not surprisingly, therefore, a wound has been compared to a tumor that heals itself (Haddow, 1972; Dvorak, 1986).

Immunohistochemical studies using TGF-β1 antibody in the developing mouse embryo suggest that TGF-β1 functions in the remodeling of embryonic tissue (Heine *et al.*, 1987). Staining with the TGF-β1 antibody was most intense during morphogenesis of mesenchyme derived tissues, such as connective tissue, cartilage and bone. This correlates well with the role of TGF-βs in wound healing and tissue repair, where TGF-βs can serve as potent stimulators of extracellular matrix formation, angiogenesis and growth stimulators of fibroblasts and osteoblasts. Intradermal treatment with TGF-β induces the

formation of granulation tissue (Roberts *et al.*, 1986), which is a characteristic phase during wound repair (Chapter 10). Strikingly, granulation tissue and tumor stroma consist of the same elements, including inflammatory cells, neovasculature and connective tissue (Roberts *et al.*, 1988). Tumor cells often secrete higher levels of TGF-β than their normal cellular counterparts (Derynck *et al.*, 1987; Jakowlew *et al.*, 1988; Niitsu *et al.*, 1988) which may, via paracrine effects, stimulate stromal elements surrounding the tumor and thereby create a favorable microenvironment for rapid tumor growth. In rouse sarcoma virus(RSV)-infected chickens wounding can act as a tumor promoter with TGF-β being the mediator in this process (Sieweke *et al.*, 1990). In the RSV-infected chickens, TGF-β is present locally after wounding (and not in control tissue) and subcuteneous injected TGF-β in RSV-infected chickens can substitute for wounding, leading to tumor formation (Sieweke *et al.*, 1990). Additionally, TGF-βs may play roles in tumorigenesis in other manners. Some tumor cells have lost their growth inhibitory responsiveness to TGF-βs (Chapter 6; Keller *et al.*, 1989; Kimchi *et al.*, 1988) which may lead to abnormal growth. TGF-βs may also contribute to tumorigenesis and metastasis by their potent immunosuppressive activity (Kehrl, *et al.*, 1986 a, b). The secretion of TGF-β by tumor cells may decrease the host immune surveillance. Glioblastoma patients are severely immune suppressed, and the causative agent appears to be high levels of TGF-β2 secreted by the tumor cells (Wrann *et al.*, 1987).

Growth factors and their cellular response systems appear to play a key role as signaling agents in embryogenesis, wound healing as well as cancer. As the underlying mechanisms are the same, the results from one field of study may lead to a concomitant understanding of the other research areas.

Therapeutic Applications of Growth Factors

TGF-βs as therapeutic agents, show promise for multiple conditions, including wound healing, bone repair, immunosuppression, anti-inflammation and as adjuvant cancer therapy for chemo- and radioprotection (Chapters 3 and 10). Clinical trials with TGF-βs are underway for treatment of psoriasis and ulcers. Currently, we are evaluating the use of TGF-β3 to reduce chemotherapy associated cytotoxicity. In the treatment of neoplastic disease the toxicity of chemoterapeutic drugs to the hematopoietic system is a major limiting factor. In collaboration with Bayard Clarkson and colleagues (Memorial Sloan-Kettering Cancer Center, USA) we found that TGF-β3 at pico Molar concentrations completely inhibits the growth of human hematopoietic stem cells (Strife *et al.*, 1990). Growth inhibition of over twenty different tumor cell lines, however, required a dose of TGF-β3 that is approximately three orders of magnitude higher. Some tumor cells did not respond to TGF-β at all (K.K. Iwata and P. ten Dijke, unpublished results and Chapter 6). Assuming this is not an artifact due to the differences between primary cells and cultured cell lines, it should be possible to use TGF-β3 to protect the stem cell proliferation *in vitro*, while using cell-cycle specific cytotoxic agents to purge the bone marrow cells of residual tumor cells prior to the autologous bone marrow transplants. Recently, Goey *et al.*. demonstrated that TGF-β1, injected as a single bolus into the femoral artery of mice (5 μg/mouse) produced a suppression of multipotential progenitor cells, in a reversible manner, suggesting that TGF-βs may prove useful as chemoprotective agents *in vivo*. If this were possible in cancer patients, TGF-βs could dramatically decrease the side effects of conventional chemotherapy, as well as allowing the implementation of a significant more aggressive chemotherapeutic regimen.

A small number of growth factors have already entered the clinic and shown to be efficacious, sometimes with no significant side effects (Bronchud and Dexter, 1989). Many

more growth factors are being evaluated as potential therapeutics. However, it will entail a further understanding of the molecular and cellular mechanisms that determine their (patho)-physiological roles and pharmacological activity before most growth factors can be used as therapeutic agents.

As growth factors act in combinations *in vivo*, an effective cocktail of growth factor may exist for specific therapeutic indications. Moreover, the presence of any one specific growth factor may only be required at defined times as the differentiation state and microenvironment changes during treatment. Furthermore, understanding the mode of action of each effect of multifunctional growth factor through structure-function studies may allow for the design of analogs with a more restricted action range. Alternatively, growth factor analogs (receptor antagonists) can possibly be identified that bind but do not activate the receptor thereby blocking growth factor action. Analysis of growth factor binding sites with inactivating (scavenger) proteins may lead to analogs with a prolonged *in vivo* half life and overcome problems with high or multiple dose requirements. Additionally, the growth factor (receptors) may be used as research tools in high throughput screens for synthetic drug discovery of agonists or antagonists. Thus, we can use molecular biological techniques not only to isolate and produce large amounts of growth factors, but also to modify growth factors or to format them into drug screens to obtain more effective pharmaceuticals. In the near future, growth factor-based therapies are likely to have a major impact on medicine and may replace many existing therapies and provide solutions to illnesses that are currently not amenable to effective treatment.

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CHAPTER TWELVE

Summary/Samenvatting

SUMMARY

During development and tissue homeostasis, an intricate communication network exists to control cell growth and differentiation. Polypeptide growth (inhibitory) factors play an important part in inter-cellular signaling and trigger a complex intracellular transduction cascade, which ultimately leads to the nucleus and affects the expression of target genes. The induction or repression of these genes will determine if the cell divides or remains quiescent. A number of growth factors and their signal transducing mediators are products of either proto-oncogenes or anti-oncogenes.

Recent years have seen a rapid accumulation of information on the role of growth-stimulating regulators, while limited progress has been made to identify growth inhibitors. The aim of the research described in this thesis was to identify and study human growth inhibitory proteins, which could lead to a better understanding of the mechanisms of normal growth control. Additionally, such information could result in new strategies for diagnosis, therapeutic intervention and prevention of growth related diseases. This thesis, in particular, describes the cloning and characterization of one such growth inhibitor, transforming growth factor-β3 (TGF-β3). However, as with many other growth regulators, TGF-β3 exhibits multifunctional properties, such as stimulating cell growth and affecting other cellular functions.

Chapters 2 and 3 review the molecular aspects of growth control and the TGF-βs, respectively. Chapters 4 through 9 describe the experimental work on TGF-β3: molecular cloning, chromosomal localization, recombinant expression, protein purification, biological characterization and receptor binding. The therapeutic applications of growth factors for wound healing are discussed in Chapter 10.

The TGF-βs are a family of dimeric 25 kDa proteins (three homodimeric isoforms in mammals), with widespread effects on many cell types, including regulation of proliferation and differentiation and stimulation of extra cellular matrix formation. Physiologically, they are thought to play a role in multiple processes, including the morphogenetic events during embryogenesis, wound healing and bone remodeling (Chapter 3).

The third member of the TGF-β family was identified from cDNA libraries by differential hybridization with a TGF-β1 cDNA probe. The TGF-β3 cDNA revealed an open reading frame encoding a protein of 412 amino acids, with strong similarity to TGF-β1 and TGF-β2 sequences. By analogy to TGF-β1 and TGF-β2, we predicted the TGF-β3 precursor to be proteolytically processed to a C-terminal mature segment of 112 amino acids. The N-terminal precursor segment contains four potential glycosylation sites and a tetra peptide RGDL, which could play a role in cellular adhesion. At the N-terminus there is a stretch of hydrophobic amino acid residues that probably correspond to the core of the signal peptide, as is found for TGF-β1 and TGF-β2. Northern analysis indicates that TGF-β3 is expressed as a single mRNA species of ~3.5 kb in multiple cell lines and tissues, including umbilical cord (Chapter 4).

TGF-β3 is highly conserved in evolution, as the presence of a related single copy gene was detected in a wide range of animal species. The same high degree of conservation is found for TGF-β1 and -β2, suggesting that all TGF-βs play essential roles in physiology. The TGF-β3 gene was mapped to human chromosome 14 by Southern blot analysis of human-Chinese hamster somatic cell hybrid lines using a probe specific for TGF-β3. Using *in situ* hybridization of human metaphase chromosomes, the regional location of TGF-β3 on chromosome 14 to bands q23-q24, was identified. All members of TGF-β superfamily have been mapped on different chromosomes (or arms) suggesting that after gene amplification, these genes have dispersed and subsequently diverged in sequence during evolution (Chapter 5).

Recombinant TGF-β3 was expressed in Chinese Hamster Ovary (CHO) cells, using dihydrofolate reductase (*dhfr*) gene amplification. Using TGF-β3 peptide antibodies, it was shown that in the conditioned media of transfected cells, both the mature as well as precursor forms of TGF-β3 were present. The recombinant TGF-β3 protein was purified to apparent homogeneity using immunoaffinity chromatography. TGF-β3 stimulated the growth of normal rat kidney (NRK) cells in soft agar (in the presence of epidermal growth factor) and inhibited the growth of multiple tumor cell lines (Chapter 6).

TGF-β3 was also shown to be a potent regulator of functions associated with bone formation, ie. mitogenesis, collagen synthesis and alkaline phosphatase activity in osteoblast-enriched bone cell cultures. TGF-β3 has somewhat more potent effects than TGF-β1 on these cells. Crosslinking experiments with iodinated TGF-β showed that in osteoblasts, both TGF-β3 and TGF-β1 associate with three cell surface binding proteins (also termed receptor types I, II and betaglycan) with affinities in the pico Molar range (Chapter 7).

Comparing the biological activity of the three mammalian homodimeric isoforms revealed that TGF-β3 is 4 fold more potent than TGF-β1 and TGF-β2 as a growth inhibitor of the Mv1Lu mink lung epithelial cell line. However, in the FBHE fetal bovine heart endothelial cell line, TGF-β1 and TGF-β3 are at least 50-fold more potent than TGF-β2 as growth inhibitors. TGF-βs differ in their binding to receptor types I and II, which are implicated in signal transduction. TGF-β1 and TGF-β3 are much more potent than TGF-β2 as competitors for binding of ^{125}I-labeled TGF-β1 to receptors I and II, which does not correspond with their potency on mink cells. We demonstrated the presence of a subset of type I and II receptors that have a high affinity for TGF-β2 in mink cells, but not in FBHE cells, that may lead to cell-specific differences in responsiveness to this isoform (Chapter 8).

TGF-β3 induced the mesoderm formation in explants of *Xenopus* blastula ectoderm, and it was found to be less potent than TGF-β2, whereas TGF-β1 alone showed no mesoderm induction. TGF-β3 showed synergism with basic FGF and was more potent than TGF-β1 in this respect. The result that TGF-βs can substitute for amphibian mesoderm induction signals suggests a role for these factors in mammalian early development (Chapter 9).

The *in vitro* effects of growth factors (including TGF-βs) suggest many potential therapeutic applications, including tissue regeneration, wound healing, immunomodulation and for the treatment of cancer. Some growth factors are already in clinical trials and have been shown to be efficacious (Chapters 10 and 11).

SAMENVATTING

De cellulaire groei en differentiatie worden tijdens de embryonale ontwikkeling en weefsel-homeostasis gecontroleerd door een ingewikkeld communicatienetwerk. Een belangrijke rol in de inter-cellulaire signaalgeving wordt vervuld door polypeptide groeifactoren. Deze factoren kunnen na cellulaire uitscheiding met sterke affiniteit zich specifiek binden aan de op het celoppervlak gelegen herkenningseiwitten (receptoren) en vervolgens kunnen ze een ingewikkelde intra-cellulair transductie-cascade induceren. De signalen bereiken uiteindelijk de celkern, waar de expressie van een selectief aantal genen verandert. De inductie of repressie van deze genen is bepalend of celdeling optreedt, of de cel in een rustende toestand blijft. Een aantal bemiddelaars, betrokken in de inter-en intra-cellulaire signaal-mechanismen, zijn producten van proto-oncogenen of anti-oncogenen.

In de laatste jaren is een grote hoeveelheid informatie verzameld over de rol van groeistimulerende factoren. Er is echter weinig voortgang gemaakt bij het onderzoek naar factoren betrokken zijn bij de groeiremming. De identificatie en karakterisering van groeiremmers is noodzakelijk voor een beter begrip van de regulatie-mechanismen betrokken bij de normale groei. Bovendien kan deze kennis mogelijk worden toegepast in de ontwikkling van nieuwe strategieën voor het stellen van de diagnose, therapeutische interventie en preventie van groei-gerelateerde ziekten. Dit proefschrift beschrijft de klonering en karakterisering van een dergelijke groeiremmer, transforming growth factor-β3 (TGF-β3). TGF-β3 heeft echter niet alleen een groeiremmende activiteit, maar vertoont zoals veel andere groeiregulatoren een multi-functioneel gedrag.

De hoofdstukken 2 en 3 geven respectievelijk een overzicht van de moleculaire aspecten van groeiregulatie en TGF-β's. De hoofdstukken 4 tot en met 9 beschrijven het uitgevoerde experimentele werk aan TGF-β3 : de moleculaire klonering, chromosomale lokalisering, recombinante expressie, eiwitzuivering, biologische karakterisering als een groei- en differentiatie-regulator en de receptorbinding. In hoofstuk 10 worden tenslotte de therapeutische toepassingen van groeifactoren voor wondheling behandeld.

TGF-β's vormen een familie van dimere 25 kDa eiwitten (drie homodimere isovormen in zoogdieren) met een grote verscheidenheid aan biologische effecten, waaronder de regulering van celdeling en differentiatie en de stimulering van extracellulaire matrix-vorming. TGF-β's vervullen mogelijk een functie in meerdere fysiologische processen, waaronder de morphologische gebeurtenissen tijdens de embryogenese, wondheling en de opbouw en afbraak van been (hoofdstuk 3).

Het derde lid van de TGF-β familie werd geïsoleerd uit een cDNA-bank door differentiele hybridisatie met een TGF-β1-probe. Het TGF-β3 cDNA bevat een open leesraam, koderend voor een eiwit van 412 aminozuren met sterke sequentie-homologie met TGF-β1 en TGF-β2. We voorspelden, naar analogie van TGF-β1 en TGF-β2, dat het TGF-β3 precursor-eiwit proteolytisch wordt gesplist tot een carboxy terminaal biologisch actief segment van 112 aminozuren. Het amino-terminale uiteinde bevat vier potentiele glycosyleringsplaatsen en een tetrapeptide RGDL, hetgeen een rol zou kunnen spelen in de cellulaire adhesie. Het amino-terminale uiteinde is rijk aan hydrofobische aminozuren en correspondeert waarschijnlijk met een signaal-peptide, analoog aan TGF-β1 en TGF-β2. Northern blot analyse van diverse cellijnen en weefsels geeft aan dat de TGF-β3 in deze cellen gekodeerd is door een enkel mRNA-transcript van 3.5 kb (hoofdstuk 4).

Het TGF-β3 gen werd gelokaliseerd op het menselijke chromosoom 14 door

Southern blot analyse van mens-chinese hamster somatische cel-hybriden, gebruikmakend van een specifieke probe voor TGF-β3. Met behulp van *in situ* hybridisatie met een TGF-β3-probe op humane metafase chromosomen werd de regionale locatie van TGF-β3 op chomosoom 14, band q23-q24, bepaald. Alle leden van de TGF-β familie liggen op verschillende chromosomen, wat erop wijst dat na genvermeerdering deze genen zijn verspreid en gedurende de evolutie vervolgens in sequentie gedivergeerd zijn (hoofdstuk 5).

Recombinant TGF-β3 werd tot expressie gebracht in eierstok cellen van een chinese-hamster (CHO), gebruikmakend van dihydrofolate reductase (*dhfr*) genvermeerdering. Met behulp van TGF-β3 peptide-antilichamen werd aangetoond dat geconditioneerd media van getransfecteerde cellen, zowel de gesplitste (25 kDa), alswel de precursor (100 kDa) vorm van TGF-β3 bevatte. Het recombinant TGF-β3 eiwit werd gezuiverd tot ogenschijnlijk homogeniteit, gebruikmakend van immuno affiniteits-chromatografie. Gezuiverd TGF-β3 stimuleert de groei van normale niercellen van een rat in zachte agar (in aanwezigheid van epidermal growth factor) en remt de groei van meerdere tumorcellijnen (hoofdstuk 6).

TGF-β3 is ook een sterke regulator van functies, geassocieerd met been-vorming, zoals celproliferatie, collageenvorming en basische fosfatase-activiteit. In osteoblast verrijkte beencelcultures blijkt TGF-β3 iets meer biologisch actief te zijn dan TGF-β1. Kruisbinding-experimenten met radioactief gelabelde TGF-β's geven aan dat in osteoblasten zowel TGF-β3 alswel TGF-β1 associëren met drie membraaneiwitten (receptor types I en II en betaglycan) met een affiniteit in de orde van pico-molair (hoofdstuk 7).

Vergelijking van de biologische activiteit tussen de drie zoogdier homodimere TGF-β isovormen geeft aan dat voor Mv1Lu minklong-epitheelcellen TGF-β3 een 4 keer zo grote groeiremmende werking heeft als TGF-β1 en TGF-β2. TGF-β1 en TGF-β3 remmen echter de groei van FBHE foetale-koehart-endotheelcellen 50 keer zo sterk als TGF-β2. De TGF-β's verschillen in affiniteit voor de receptortypes I en II, die zijn betrokken in signaal-doorgeving. TGF-β1 en TGF-β3 zijn meer kompetitief dan TGF-β2 van de receptorbinding van ^{125}I-gelabeled TGF-β1, hetgeen niet correspondeert met hun relatieve groeiremmende activiteit voor mink-cellen. Wij hebben aangetoond aan dat een bepaalde subgroep van type I en II receptoren met een sterke affiniteit voor TGF-β2 aanwezig is in mink-cellen, maar niet in FHBE-cellen. Dit zou de celspecifieke verschillen in gevoeligheid voor deze isovormen kunnen verklaren (hoofdstuk 8).

TGF-β3 vertoont ook mesoderm-inducerende activiteit voor cellen van *Xenopus* blastula-ectoderm. TGF-β3 blijkt hierin minder actief te zijn dan TGF-β2. TGF-β1 alleen vertoont geen mesoderm-inducerende activiteit. Zowel TGF-β1 alswel TGF-β3 vertonen synergie met basische FGF,waarbij TGF-β3 meer biologisch actief is dan TGF-β1. De waarneming dat de TGF-β's mesoderm kunnen induceren in *Xenopus*, wijst ook op een mogelijke rol voor deze factoren in de vroege embryonale ontwikkeling van zoogdieren (hoofdstuk 9).

De *in vitro* effecten van groeifactoren (waaronder TGF-β's) wijzen mogelijk op verschillende therapeutische toepassingen, waaronder weefselregeneratie, wondheling, immuno-modulatie en op de genezing van kanker. Sommige groeifactoren worden inmiddels al toegepast in de kliniek en blijken effectief te zijn zonder nadelige neveneffecten te vertonen (hoofdstukken 10 en 11).

CURRICULUM VITAE

Peter ten Dijke werd op 4 September 1960 te Enschede geboren. In 1979 behaalde hij het diploma VWO-B aan de Scholengemeenschap Zuid te Enschede. In hetzelfde jaar begon hij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. In 1983 behaalde hij het kandidaatsdiploma in deze studierichting. De doctoraalstudie, die cum laude werd afgesloten in januari 1987, omvatte drie hoofdvakken: Biochemie (Prof. Dr. C. Veeger) en Moleculaire Biologie (Prof. Dr. A. van Kammen) aan de Landbouwuniversiteit te Wageningen en Cel Biologie (Prof. Dr. D. Bootsma) aan de Erasmusuniversiteit te Rotterdam.

Zijn interesse voor toegepast kankeronderzoek heeft geleid tot een stageplaats bij het amerikaanse bedrijf Oncogene Science, gevestigd in New York. Vanaf januari 1986 tot heden is hij in dienst als stafwetenschapper bij dit bedrijf. Aanvankelijk was hij, onder leiding van Drs. Nora Heisterkamp en John Groffen betrokken bij het onderzoek naar oncogenen met toepassing in de kankerdiagnostiek. In het kader van een samenwerkingsverband tussen de bedrijven Oncogene Science en Pfizer, met als doel de ontwikkeling van betere geneesmiddelen voor kanker, werd het verrichte onderzoek beschreven in dit proefschrift.