Connective Tissue Formation in Wound Healing

An experimental study

Kaija Inkinen

Fourth Department of Surgery
Helsinki University Central Hospital
and
Division of Biochemistry
Department of Biosciences
University of Helsinki
Finland

Supervised by

Professor Juhani Ahonen
Department of Surgery
Helsinki University Central Hospital
Helsinki, Finland

Reviewed by

Professor Jorma Keski-Oja
Departments of Pathology and Virology
Biomedicum and Haartman Institute
University of Helsinki
Helsinki, Finland

and

Docent Erkki Koivunen
Department of Biosciences
Division of Biochemistry
University of Helsinki, Finland

Opponent

Docent Jouko Lohi
Department of Pathology
University of Helsinki
Helsinki, Finland

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV.

I. Inkinen, K. Turakainen, H. Wolff, H. and Ahonen, J.: Cloning of cDNA for rat pro alpha1(V) collagen mRNA. Expression patterns of type I, type III and type V collagen genes in experimental granulation tissue. **Connective Tissue Research.** 40:209-220, 1999

II. Inkinen, K. Wolff, H. von Boguslawski, K. and Ahonen, J.: Type V Collagen in Experimental Granulation Tissue. **Connective Tissue Research.** 39:281-294, 1998

III. Inkinen, K. Turakainen, H. Wolff, H. Ravanti, L. Kähäri, VM. and Ahonen, J.: Expression and activity of matrix metalloproteinase-2 and -9 in experimental granulation tissue. **Acta Pathologica, Microbiologica, et Immunologica Scandinavica.**108:318-328, 2000

IV. Inkinen, K. Wolff, H. Lindroos, P. and Ahonen, J.: Connective tissue growth factor and its correlation to other growth factors in experimental granulation tissue. **Connective Tissue Research.** 44: 19-29, 2003

ABBREVIATION

AP-1	activator protein-1
CCN	Connective tissue growth factor/Cysteine-rich 61/Nephroblastoma over-expressed
CTGF	connective tissue growth factor
ECM	extracellular matrix
EGF	epidermal growth factor
Egr-1	early growth response 1
FACIT	fibril-associated collagens with interrupted triple helices
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
ICAM-1	intercellular adhesion molecule 1
IFN γ	interferon γ
IGF-1	insulin-like growth factor 1
IGF-BP	insulin-like growth factor binding protein
IL-1, -2, -4, -6	interleukin 1, 2, 4, 6
KGF	keratinocyte growth factor
LAP	latency associated protein
LTBP	latent TGF-β binding protein
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase
MULTIPLEXIN	proteins with multiple triple helix domains and interruptions
NF-1	nuclear factor-1
PAGE	polyacrylamide gel electrophoresis
PARP	proline and arginine rich peptide
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDI	protein disulfide isomerase
PGE_2	prostaglandin E_2
SMC	smooth muscle cell
Sp-1	selective promoter factor 1
TbRE	TGF-β-response element
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor-α
VC	viscose cellulose
VEGF	vascular endothelial growth factor

ABSTRACT

A tight balance between connective tissue synthesis and breakdown is required for extracellular matrix (ECM) deposition in normal wound healing. The formation of new and regenerating tissue requires the coordinated regulation of various genes, which encode both structural and regulatory molecules. In the present study, the spatial and temporal expression patterns of various key components in the connective tissue formation of normal wound healing were investigated.

Type I, III and V collagens belong to the family of fibrillar collagens, which form the important structural component of connective tissue. In the present study, type V collagen, in relation to type I and III collagens, in the formation of new connective tissue was investigated. Type V collagen was actively synthesized by both macrophage and fibroblasts-like cells and its expression was seen at every time point but at a considerably lower level than with type I and III collagens. It had maximum expression level at two weeks, which coincides with the expression of myofibroblasts. At the protein level, its distribution was closely associated with the blood vessel walls, which suggests its association with angiogenesis.

From the collagen degrading enzymes, gelatinases of the matrix metalloproteinases (MMP) family, MMP-2 and MMP-9 were investigated. In addition, the expression of MMP-14 and tissue inhibitor of matrix metalloproteinase 2 (TIMP), which are known to be important for the activation of latent MMP-2, were studied. Enzyme analyses revealed that latent MMP-2 was present during the whole period of the granulation formation, whereas active MMP-2 started increase after one week and stayed in high level during the whole period of experiment. MMP-2 mRNA was expressed throughout the granulation formation and mostly in fibroblast-like cells. The MMP-14 gene was up-regulated during the first week of wound healing, which coincides with an increase in MMP-2 activation. In contrast, the TIMP-2 gene was constitutively expressed, as was the MMP-2 gene. Only after two months was the TIMP-2 mRNA level slightly higher than at other time points, implying the completion of the wound healing process. No active MMP-9 enzyme was found and latent MMP-9 was seen only during the first week of the healing process. Immunohistochemical staining revealed that the cells responsible for this MMP-9 were polymorphonuclear leukocytes and macrophages, which are probably invaders from the surrounding tissues and already have pro-enzyme in their granules. MMP-9 mRNA expression started to increase towards the end of the experiment but with no detectable concomitant enzymatic activity. This suggests that MMP-9 regulation occurs at the

post-transcriptional level. The most prominent cell types to express MMP-9 mRNA were macrophage-like cells.

Connective tissue growth factor (CTGF) is downstream mediator of transforming growth factor-β (TGF-β) in fibroblasts. For the elucidation of its role in normal wound healing, its gene expression and protein localization, together with other fibrogenic growth factors, TGF-β1, and platelet-derived growth factors (PDGF), were studied. All these growth factors were found to be expressed throughout the wound healing process but CTGF had a more restricted expression pattern. CTGF mRNA had maximum expression at two weeks and the expressing cells were mostly fibroblasts, but in the early phase of wound healing also the blood vessel cells expressed CTGF at the gene and protein levels, suggesting that it is involved in angiogenesis.

In conclusion, successful wound healing is accompanied by tightly scheduled expression of fibrillar collagens, matrix metalloproteinases and growth factors. The close association of type V collagen and CTGF in blood vessels during wound healing suggests their role in angiogenesis. MMP-2 is important during the prolonged remodeling phase, whereas the MMP-9 gene is up-regulated when the granulation tissue matures. MMP-2 and MMP-9 might facilitate an essential event of wound healing, such as cell migration, angiogenesis and tissue remodeling.

INTRODUCTION

The regulation of extracellular matrix (ECM) deposition is a key event in many physiological and pathological conditions. It is required for normal wound healing where ECM molecules need to be rapidly synthesized during the formation of early granulation tissue and also during the final replacement by mature connective tissue and tissue remodeling. A tight balance between connective tissue synthesis and breakdown is therefore required for the normal functioning of all tissues. The formation of new and regenerating tissue requires the coordinate regulation of various genes, which encode for both structural and regulatory molecules that participate in cell growth and tissue organization.

Collagens are the major macromolecules of connective tissues and the most abundant proteins in the human body. Besides their structural role in numerous tissue and organs, collagens have a number of other important biological functions, such as cell attachment, chemotaxis and platelet aggregation. Fibril-forming collagens in soft tissues are type I, III and V. Type I collagen is the major collagen in most tissues. Type III collagen occurs in tissues exhibiting elastic properties, such as skin and blood vessels. Type V collagen is a quantitatively minor fibrillar collagen with a broad tissue distribution.

Degradation of ECM proteins is essential in tissue repair where cell migration is an important event. The matrix metalloproteinase (MMP) family is a group of proteases with different ECM substrate specificities. Of these, MMP-2 and MMP-9 form the gelatinase sub-family. These enzymes have the capacity to degrade types IV and V collagen and the degradation products of collagens and elastin. Metalloproteinases are secreted as pro-enzymes, which undergo extracellular activation. The extracellular activity of these enzymes is modulated also by interaction with the specific tissue inhibitors of metalloproteinases (TIMP), and microenvironmental factors.

Growth factors are essential for regulating the molecular and cellular events involved in the formation of granulation tissue and in wound healing. Growth factors affect the different phases in wound healing differently. Various growth factors induce their own synthesis in positive autocrine feedback loops, as well as of other growth factors. Thus, a plethora of factors is likely to be present at the site of the wound, which assures efficient enhancement of the different phases of wound healing. The most important granulation modulating growth factors known so far are platelet-derived growth factors (PDGF's), transforming growth factor β (TGF-β) and connective tissue growth factor (CTGF).

The present study was carried out to examine temporal expression of types I, III and especially type V collagens in developing granulation tissue. To understand the process, the gene expression and enzyme activity of metalloproteinases MMP-2 and MMP-9 were studied. These enzymes degrade type V collagen and gelatins of other collagen types. CTGF is a downstream mediator of TGF-β in fibroblasts. For the elucidation of its role in normal wound healing, its gene expression and protein localization together with other fibrogenic growth factors, TGF-β1 and PDGF's were studied.

REVIEW OF THE LITERATURE

WOUND HEALING

Wound healing is a complex and dynamic cascade of events initiated by injury. This response to injury is a phylogenetically primitive, yet essential, innate host immune response for the restoration of tissue integrity (*1, 2*). The processes involve coordinated cell activation, cell division, chemotaxis and migration, and differentiation of many cell types. They are mediated by locally released growth factors and cytokines, which may act in an autocrine or paracrine manner. All phases of wound healing are either directly or indirectly controlled by cytokines. It is the balance of these cytokines and other mediators, rather than the mere presence or absence of one or more cytokines, which plays a decisive role in regulating the initiation, progression and resolution of wounds (*3-6*). In addition, cell-cell and cell-matrix interactions, mediated, for
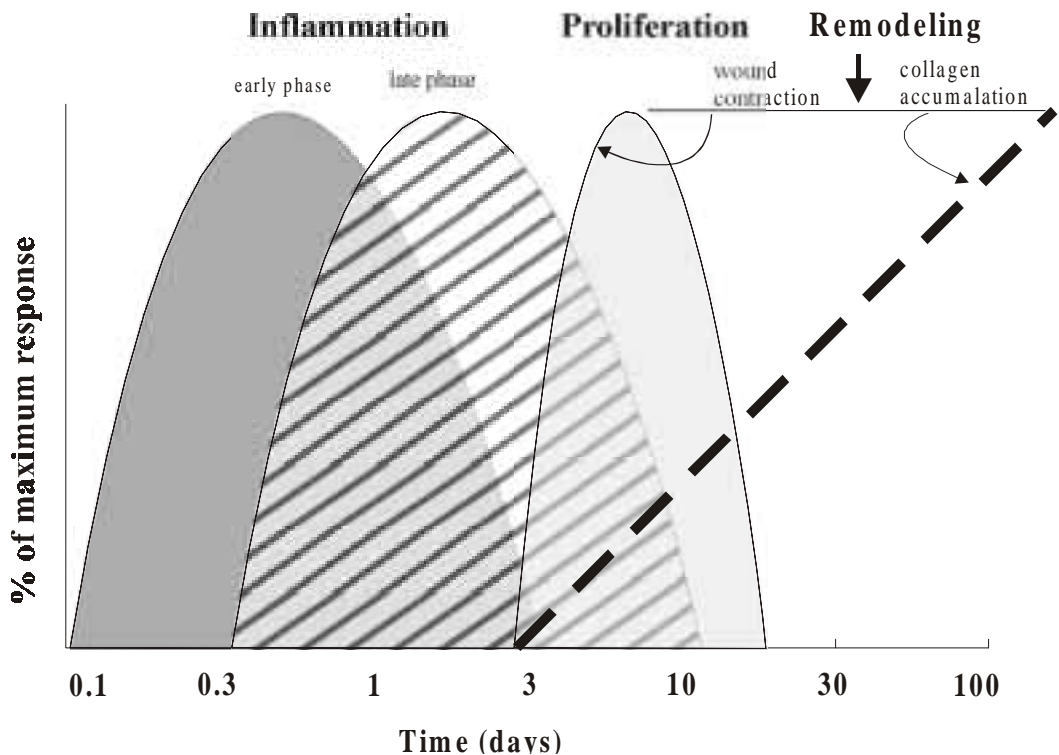
Figure 1. Phases of cutaneous wound repair. Healing of a wound has been divided into three phases: inflammation (early and late), proliferation, and remodeling (*7*). These wound repair processes are plotted along the abscissa as a logarithmic function of time. The phases of wound repair overlap considerably with one another. Inflammation is divided into early and late phases denoting neutrophil-rich and mononuclear cell-rich infiltrates, respectively. Modified from (Clark, 1996) (*7*)

example by various cell surface adhesion molecules, play an important role in wound healing. The balance of pericellular proteases is also important. Wound healing may be divided into distinct phases, as characterized by both the predominant cellular population and cellular function. Irrespective of the affected tissue, the wound healing process follows a conserved sequence of events which overlap in time, including inflammation, tissue formation and tissue remodeling (Fig. 1.). In normal wound healing, a network of negative feedback mechanisms, activated after successful healing, is responsible for the proper termination of the proliferative and fibrotic processes, thus restoring tissue integrity (*1, 2, 7*).

Blood clot formation
Tissue injury causes the disruption of blood vessels and extravasation of blood constituents. The blood clot re-establishes hemostasis and provides a provisional extracellular matrix for cell migration (*2*). The clot consists of platelets within a network of crosslinked fibrin fibers, derived by thrombin cleavage of fibrinogen, together with smaller amounts of plasma fibronectin, vitronectin and thrombospondin (*7*). Among the important functions of the clot are its role as a reservoir of growth factors and cytokines that are released by the granulation of activated platelets. Growth factors such as transforming growth factor (TGF) -α and -β, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF)-1 are involved in tissue repair initiation as potent chemotactic and mitogenic molecules for inflammatory and connective tissue cells (*3, 8-10*).

Inflammation
Chemotactic signals attract neutrophils and monocytes to wound sites (*11*). Besides growth factors released by platelets, other cues, such as peptides cleaved from bacterial proteins and the by-products of proteolysis of fibrin and other matrix components, act as chemotactic signals (*12*). Both neutrophils and monocytes are recruited from the circulating blood in response to molecular changes in the surface of endothelial cells lining capillaries at the wound site. Neutrophils normally begin arriving at the wound site within minutes of injury; their role being the clearance of the initial rush of contaminating bacteria, but neutrophils are also a source of pro-inflammatory cytokines that probably serve as some of the earliest signals to activate local fibroblasts and keratinocytes (*13*). The neutrophil infiltration ceases after a few

days, and neutrophils are themselves phagocytosed by tissue macrophages. Macrophages continue to accumulate at the wound site by recruitment of blood-borne monocytes and are essential for effective wound healing; if macrophage infiltration is prevented, then healing is severely impaired (*14*). Macrophage tasks include phagocytosis of any remaining pathogenic organisms and other cell and matrix debris. Once activated, macrophages also release a battery of growth factors and cytokines at the wound site, thus amplifying the earlier wound signals released by degranulating platelets and neutrophils (*1*).

Epithelialization
Re-epithelalization of wound begins within hours after injury (*1, 2*). The cells undergo marked phenotypic alteration. In unharmed skin, the basal layer of epithelium is attached to a specialized matrix, the basal lamina. Integrins are transmembrane heterodimers of α and β subunits that bind to the extracellular matrix (ECM) through a large extracellular domain. Keratinocytes in the epithelium use the integrins to bind to laminin in the basal lamina, and these integrins have intracellular links with the keratin cytoskeletal network. The keratinocytes at the edge of the surgical wound have to dissolve the hemidesmosome attachment and begin to express other integrins that are more suitable for the wound environment. The changes in the expression of integrins by cells are important for cell migration in the healing wound. Epidermal movement through tissues depends on epidermal cell production of collagenolytic enzymes and plasminogen activator. The mechanisms that drive epithelial cell migration may be chemotactic factors, active contact guidance, absence of neighboring cells or a combination of these processes. Once re-epithelialization is complete, the components of the basal lamina are deposited in a sequential manner, starting form the wound margin and the epithelial cells revert to their normal phenotype. The key growth factors in stimulating the proliferation of keratinocytes in healing wounds are the EGF, TGF-α, heparin binding epidermal growth factor (HB-EGF), hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) (*3, 4, 15, 16*). TGF-β, on the other hand, is the most important inhibiting factor for epithelial cell growth (*17*).

Neovascularization
The wound connective tissue is known as granulation tissue because of the pink granular appearance of numerous capillaries that invade the wound neodermis. Capillaries arise from blood vessels adjacent to the wound. The formation of new blood vessels is necessary to

sustain the newly formed granulation tissue (*18*). Basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) released at the wound site promote angiogenesis. Basic FGF is released by damaged endothelial cells and by macrophages (*4*). VEGF is induced in wound-edge keratinocytes and macrophages, possibly in response to KGF and TGF-α, and synchronously at least one of its receptors is up-regulated by endothelial cells at the site of injury (*19*). Basic FGF may set the stage for angiogenesis during the first days of wound repair, whereas VEGF is critical for angiogenesis during the formation of granulation tissue during later phases of wound healing (*20*). In addition to angiogenic factors, appropriate extracellular matrix and endothelial receptors for the provisional matrix are necessary for angiogenesis. Endothelial cells must up-regulate αvβ3 integrins if they are to respond to any wound angiogenic signal. Fibronectin receptor αvβ3 is expressed transiently at the tips of sprouting capillaries in the granulation tissue (*21*). Proliferating microvascular endothelial cells adjacent to and within wounds, transiently deposit increased amounts of fibronectin within the vessel walls (*22*). Perivascular fibronectin may act as a conduit for the movement of endothelial cells into the wound. After migration, basic FGF or TGF-βstimulated cells get a multicellular wall tube with lumen arrangement (*23, 24*). Pericytes then migrate along capillaries and gradually encircle the newly formed endothelium. Endothelial cells and pericytes lay down metabolic activities and organize a basal lamina made of collagen IV and laminin around the tube, to finally form the junctions between them (*24*).

Protease expression and activity are also necessary for angiogenesis (*25*). Proteolytic enzymes released into the connective tissue degrade extracellular matrix proteins. Fragments of these proteins recruit fibroblasts and peripheral-blood monocytes which become activated macrophages at the site of injury and release angiogenic factors. Basic FGF stimulates endothelial cells to release plasminogen activator and pro-collagenase (*26*). Plasminogen activator converts plasminogen to plasmin, and pro-collagenase to active collagenase and, in concert with the others, these two proteases digest the basement membranes. The fragmentation of the basement membrane allows endothelial cells stimulated by angiogenic factors to migrate and form new blood vessels at the injury site (*2*). Once the wound is filled with new granulation tissue, angiogenesis ceases and many of the new blood vessels disintegrate as a result of apoptosis (*27*). Programmed endothelial cell death is probably regulated by a variety of matrix molecules, such as thrombospondins 1 and 2 (*28*), and anti-angiogenic factors, such as angiostatin, endostatin, and angiopoietin 2 (*29*).

Formation of Granulation Tissue

New stroma, granulation tissue, begins to invade the wound space approximately four days after injury (*7*). Macrophages, fibroblasts and blood vessels move into the wound space at the same time. Macrophages provide a continuous source of growth factors necessary to stimulate fibroplasia and angiogenesis; fibroblasts produce the new extracellular matrix necessary to support cell ingrowth; and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism (*2*).

Granulation tissue is a complex reservoir of cytokines possessing chemo-attractive, mitogenic and other regulatory activities (*1*). Growth factors, especially PDGF and TGF-β1, in concert with the extracellular matrix molecules, presumably stimulate fibroblasts of the tissue around the wound to proliferate, express appropriate integrin receptors and migrate into the wound space (*30-32*). The structural molecules of newly formed extracellular matrix, termed the provisional matrix, contribute to the formation of granulation tissue by providing a scaffold, or conduit, for cell migration. These molecules include fibrin, fibronectin, and hyaluronic acid (*33, 34*). Fibroblasts have to rearrange their integrin expression profiles in preparation for migration. In normal tissues, fibroblasts reside in collagen-rich matrices. In response to injury, the fibroblasts in the vicinity of the wound have to down-regulate the integrin receptors of collagen and up-regulate those needed for adhesion to components of the provisional matrix (*32*). Fibroblasts have a remarkable ability to respond to signals from the extracellular environment; when simultaneously challenged by signals from both the provisional matrix and growth factors (such as PDGF), fibroblasts respond by up-regulating the receptors for provisional matrix components. However, when challenged by the same growth factor (PDGF) in the presence of a collagenous matrix, fibroblasts respond by up-regulating the receptors for collagen and not the provisional matrix receptors (*32*). The fibroblasts are responsible for the synthesis, deposition and remodelling of the extracellular matrix. Conversely, the extracellular matrix can have a positive or negative effect on the capacity of fibroblasts to synthesize, deposit, remodel and generally interact with the extracellular matrix (*32, 35*).

Cell movement into a blood clot of cross-linked fibrin or into tightly woven extracellular matrix, may require an active proteolytic system that can cleave a path for cell migration. A variety of fibroblast-derived enzymes, in addition to serum-derived plasmin, are potential candidates for this task, including plasminogen activator, collagenases, gelatinase A, and stromelysin (*36, 37*). After migrating into wounds, fibroblasts commence the synthesis of

extracellular matrix. The provisional extracellular matrix is gradually replaced by a collagenous matrix, perhaps as a result of the action of TGF-β1 (*38*).

Once an abundant collagen matrix has been deposited in the wound, fibroblasts stop producing collagen and the fibroblast-rich granulation tissue is replaced by a relatively acellular scar. Cells in the wound undergo apoptosis (*39*) triggered by unknown signals. Dysregulation of these processes occurs in fibrotic disorders such as keloid formation, morphea, and scleroderma (*2*).

Matrix Remodeling

Extracellular matrix remodeling, cell maturation, and cell apoptosis create the third phase of wound repair, which overlaps with tissue formation. Once the wound is filled with granulation tissue and covered with a neoepidermis, fibroblasts transform into myofibroblasts, which contract the wound, and epidermal cell differentiate to reestablish the permeability barriers. Endothelial cells appear to be the first cell type to undergo apoptosis, followed by the myofibroblasts, leading gradually to a rather acellular scar (*7*). During the proliferation phase of wound healing (Fig. 1), fibroblasts assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments disposed along the cytoplasmic face of the plasma membrane of the cells and by cell-cell and cell-matrix linkages (*38, 40*). The appearance of the myofibroblasts corresponds to the commencement of connective-tissue compaction and the contraction of the wound. The contraction probably requires stimulation by TGF-β1 or TGF-β2 and PDGF, attachment of fibroblasts to the collagen matrix through integrin receptors, and cross-links between individual bundles of collagen (*41-44*). The overall collagen content of the wound diminishes, while tensile strength increases as a result of structural modification of the newly deposited collagen, such that unorganised collagen fibrils mature into compact fibres. The increase in fibre diameter is associated within an increase in wound tensile strength. Crosslinking of collagen fibrils is largely responsible for these morphologic changes and increase in wound strength (*45*). The degradation of collagen in the wound is controlled by several proteolytic enzymes termed matrix metalloproteinases, which are secreted by macrophages, epidermal cells, and endothelial cells, as well as fibroblasts (*36*). In the various phases of wound repair, distinct combinations of matrix metalloproteinases and tissue inhibitors of metalloproteinases are needed (*46*).

During the granulation tissue formation wounds gain only about 20 percent of their final strength (*47*). During this time fibrillar collagen has accumulated relatively rapidly and has been

remodeled by contraction of the wound. Thereafter the rate at which wounds gain tensile strength is slow, reflecting a much slower rate of accumulation of collagen and collagen remodeling with the formation of larger collagen bundles and an increase in the number of intermolecular cross-links (*45*). However, this is an imperfect process since the wound collagen does not achieve the bundled, highly organized pattern seen in normal, uninjured dermis. Therefore, wounds never attain the same breaking strength (the tension at which skin breaks) as uninjured skin. At maximal strength, a scar is only 70 percent as strong as normal skin (*47*).

Viscose cellulose sponges as a wound healing model
Subcutaneous implantation of various sponge implants is a widely used method of investigating wound healing, particularly in regard to connective tissue development. Most often used sponge material for this purpose is viscose cellulose (VC). Several researchers have studied morphological and biochemical properties of the experimentally induced granulation tissue with spongy implants in the back of the rat since the early 60s (*47-50*). The development of granulation tissue in subcutaneously implanted VC sponges in rats has been shown to be similar, both histologically and chemically, to that formed in a healing wound (*47, 48, 51*).

The principle of an implanted sponge in a tissue is to create a dead space where granulation tissue can develop at the periphery. As an experimental system, the formation of sponge granulomas provides an environment of defined dimensions that is conducive to the invasion of various repair cells and the de novo formation of tissue. The histological examination of a sponge implant reveals the infiltration of inflammatory cells followed by the development of new vascularity and fibroblasts that synthesize collagen. These finding indicate the similarity between granulation tissue induced by viscose cellulose sponge and a healing cutaneous wound (*47*). The sponge model offers a well-delineated matrix with minimal irritation of the surrounding tissues and it can easily be examined at any phase of the development of granulation tissue (*47*).

Cellulose is a naturally occurring, linear homopolymer of glucose. It is insoluble in water and degradable in nature by microbial and fungal enzymes. The disappearance of cellulose in animal and human tissues is considered to be limited, if it occurs at all, because of the absence of hydrolases that attack the $\beta(1-4)$ linkage (*52*). The VC sponge has good overall homogeneity and the rate of cell invasion and tissue formation in VC sponge is rapid (*53*). Additionally, the sponge has elasticity, a property of reversible compression and expansion without damage to

the internal structure, thus providing a free entry for the cells to inner parts of the sponge (*53*). Calcification of fibrous tissue does not occur and the sponge shows negative staining properties with routine histological staining procedures (*53*). Viscose cellulose sponge implanted in wound healing studies for a short period can be regarded as a stable material with or without minimal degradation (*52*).

EXTRACELLULAR MATRIX

Collagens

Collagens are the major macromolecules of most connective tissues and the most abundant proteins in the human body. Bone, skin, tendon, cartilage, ligaments and vascular walls are particularly rich in collagens, but they are found in essentially all tissues, and play a dominant role in maintaining the structural integrity on numerous tissue and organs. In addition, collagens are involved in a number of other important biological functions, such as cell attachment, chemotaxis, platelet aggregation and filtration through basement membrane.

Collagens also play important roles in the healing of wound and fractures. On the other hand, excessive collagen formation poses a problem, leading to fibrosis in various organs and tissues (*54*).
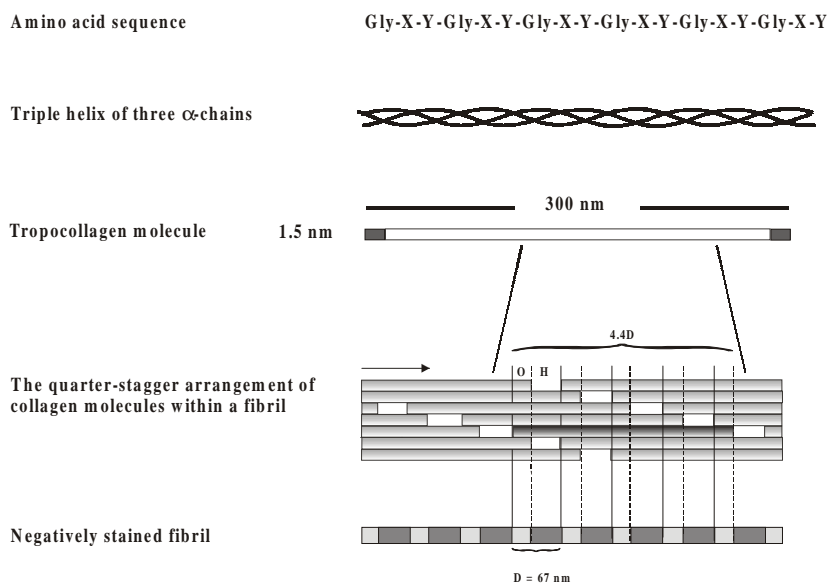

Figure 2. Structure and assembly of collagen. O = overlap zone, H = hole zone, D= the length of one cross-striation period of 67 nm. Negatively stained fibril as seen in electron microscopy. The D period is divided into a dark and a light area reflecting loosely and densely packed regions, respectively. Modified from (Eyre, 1980) (*55*).

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Table I Collagen types, their genes, molecular forms and distribution in human tissues.

Type	Constituent chains	Gene locus	Chromosomal localization	Chain composition	Subgroup	Distribution
I	$\alpha1(I)$	COL1A1	17q21.3-q22.1	$[\alpha1(I)]_2\alpha2(I)$	Fibrillar	Most tissues
	$\alpha2(I)$	COL1A2	7q22.1	$[\alpha1(I)]_3$		
II	$\alpha1(II)$	COL2A1	12q13.11-q13.2	$[\alpha1(II)]_3$	Fibrillar	Cartilage, cornea, vitreous humor, intervertebral disc
III	$\alpha1(III)$	COL3A1	2q32.2	$[\alpha1(III)]_3$	Fibrillar	Soft tissues, with type I collagen
IV	$\alpha1(IV)$	COL4A1	13q34	$[\alpha1(IV)]_2\alpha2(IV)$	Network-forming	Basement membranes
	$\alpha2(IV)$	COL4A2	13q34	$[\alpha3(IV)]_2\alpha4(IV)$		
	$\alpha3(IV)$	COL4A3	2q36-q37	other forms		
	$\alpha4(IV)$	COL4A4	2q35-q37			
	$\alpha5(IV)$	COL4A5	Xq22			
	$\alpha6(IV)$	COL4A6	Xq22			
V	$\alpha1(V)$	COL5A1	9q34.2-q34.3	$[\alpha1(V)]_3$	Fibrillar	Minor amounts in most tissues with type I collagen
	$\alpha2(V)$	COL5A2	2q14-q32	$\alpha1(V)\alpha2(V)\alpha3(V)$		
	$\alpha3(V)$	COL5A3	19p13.2	other forms		
VI	$\alpha1(VI)$	COL6A1	21q22.3	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Beaded filament-forming	Minor amounts in most tissues
	$\alpha2(VI)$	COL6A2	21q22.3			
	$\alpha3(VI)$	COL6A3	2q37			
VII	$\alpha1(VII)$	COL7A1	3p21.1	$[\alpha1(VII)]_3$	Anchoring fibril-forming	Skin, cervix, oral mucosa
VIII	$\alpha1(VIII)$	COL8A1	3q12-q13.1	$[\alpha1(VIII)]_2\alpha2(VIII)$	Network-forming	Many tissues
	$\alpha2(VIII)$	COL8A2	1p34.2-p32.3			
IX	$\alpha1(IX)$	COL9A1	6q12-q14	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	FACIT	With type II collagen, e.g. cartilage
	$\alpha2(IX)$	COL9A2	1p33-p32			
	$\alpha3(IX)$	COL9A3	20q13.3			
X	$\alpha1(X)$	COL10A1	6q21-q22	$[\alpha1(X)]_3$	Network-forming	Hypertrophic cartilage
XI	$\alpha1(XI)$	COL11A1	1p21	$\alpha1(XI)\alpha2(XI)\alpha1(II)$	Fibrillar	With type II collagen, e.g. cartilage
	$\alpha2(XI)$	COL11A2	6p21.3	other forms		
	$\alpha1(II)$	COL2A1	12q13.11-q13.2			
XII	$\alpha1(XII)$	COL12A1	6q12-q13	$[\alpha1(XII)]_3$	FACIT	Many tissues with type I collagen
XIII	$\alpha1(XIII)$	COL13A1	10q22	unknown	Transmembrane domain	Minor amounts in many tissues
XIV	$\alpha1(XIV)$	COL14A1	8q23	$[\alpha1(XIV)]_3$	FACIT	Many tissues with type I collagen
XV	$\alpha1(XV)$	COL15A1	9q21-q22	unknown	MULTIPLEXINs	Many tissues
XVI	$\alpha1(XVI)$	COL16A1	1p35-p34	$[\alpha1(XVI)]_3$	FACIT	Many tissues
XVII	$\alpha1(XVII)$	COL17A1	10q24.3	$[\alpha1(XVII)]_3$	Transmembrane domain	Hemidesmosomes of stratified squamous epithelia
XVIII	$\alpha1(XVIII)$	COL18A1	21q22.3	unknown	MULTIPLEXINs	Liver, kidney, placenta, etc.
XIX	$\alpha1(XIX)$	COL19A1	6q12-q13	unknown	FACIT	Several tissues
XX		COL20A1	Unknown	unknown		

COLLAGEN BIOSYNTHESIS

INTRACELLULAR

Proα-collagen gene

Nucleus — Transcription

Proα-collagen pre-mRNA — } Pretranslational

Nucleus — Splicing modification

Proα-collagen mRNA

Cytoplasm/RER — Translation

Preproα-collagen polypeptide

RER membrane — Signal peptide cleavage

Proα-collagen polypeptide

RER lumen — Prolyl 4-hydroxylation / Prolyl 3-hydroxylation / Lysyl hydroxylation / Hydroxylysyl glycosylation / Asparaginyl glycosylation — } Cotranslational

Proα-collagen chain

RER lumen — Proα-collagen assembly / Disulfide bonding / Triple helix formation

EXTRACELLULAR — Procollagen — Propeptide cleavage — } Post-translational

Collagen monomer — Fibril assembly / Lyslyl hydroxylysyl oxidation / Crosslinking
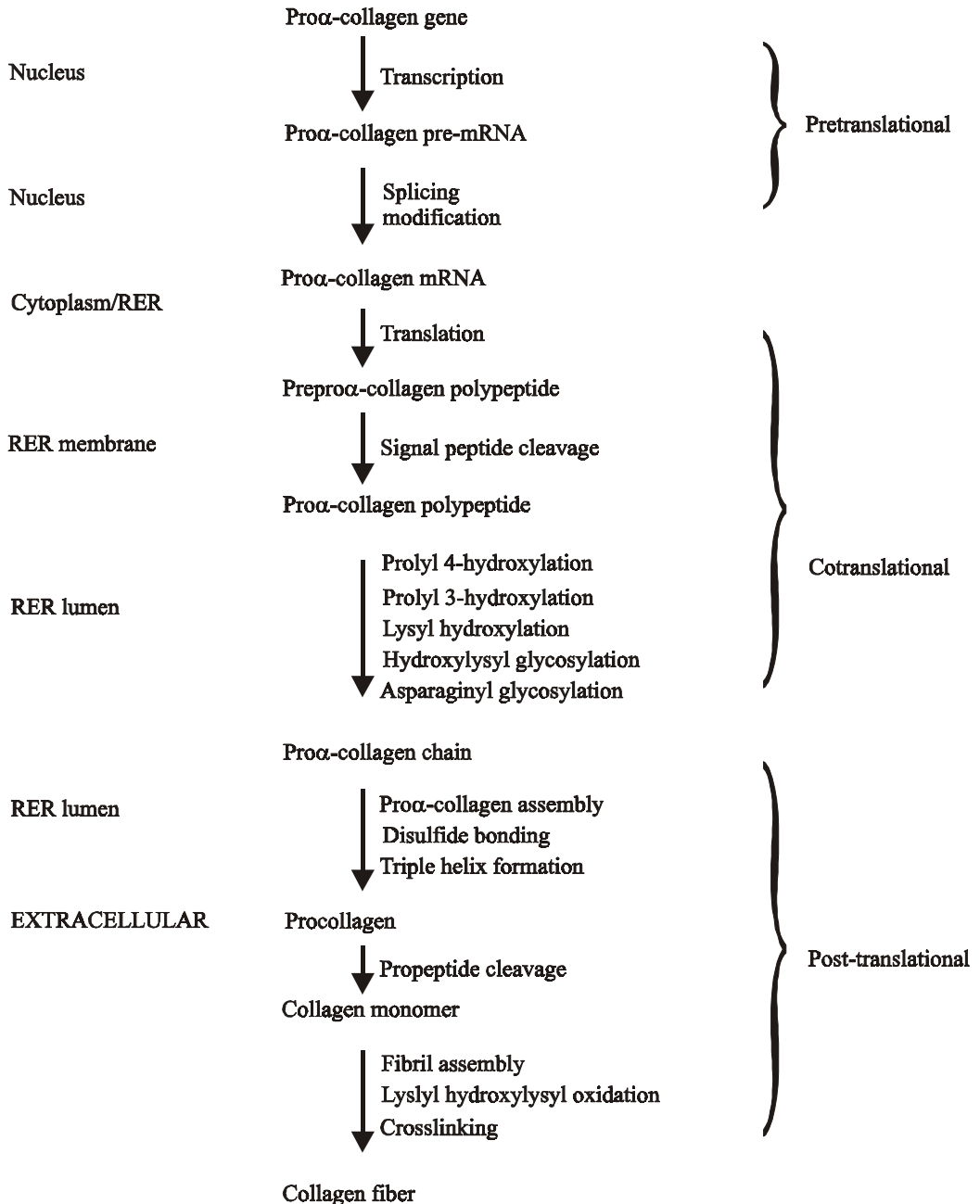
Collagen fiber

Figure 3. Steps in the biosynthesis of a collagen fiber and the location in the cell where they occur.
RER = rough endoplasmic reticulum (Phillips et al., 1992) (*56*).

Structure and biosynthesis of collagens

The characteristic feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure, in which three collagen polypeptide chains, called α-chains, are wound around one another in a ropelike superhelix. Each polypeptide forms a left-handed helix and this results in the formation of a right-handed superhelix. The amino-acid sequences of triple helical domains are characterized by the repetition of triplets Gly-X-Y. Any other amino acid sequence would perturb the triple helical conformation (Fig. 2.). The triple helical conformation is stabilized by the presence of prolyl and hydroxyproly residues in the X and Y positions and by hydrogen bonds between the chains perpendicular to the helix axis (*57*). To date at least 20 collagen types have been identified and over 30 different collagenous polypeptides, each being a distinct gene product (Table I) (*54*). Traditionally collagens have been divided into two subgroups, fibril-forming and non-fibril-forming collagens, according to their structural features. The latter group may be further divided in to subfamilies: network-forming collagens, a beaded filament-forming collagen, a collagen which forms anchoring fibrils, FACIT collagens (fibril-associated collagens with interrupted triple helices), collagens with a transmembrane domain, and MULTIPLEXINs (proteins with multiple triple helix domains and interruptions).

Collagen biosynthesis involves an unusually large number of post-translational modifications, many of which are unique to collagens and a few other proteins with collagen-like amino acid sequences. This post-translational processing takes place in two stages. Intracellular modifications, together with the synthesis of the α-chains, result in the formation of triple-helical pro-collagen molecules, and extracellular processing converts these molecules into collagens and incorporates the collagen molecules into stable cross-linked fibrils (Fig. 3.) (*58*) After transcription of the pro-collagen genes and processing of the pre-mRNAs, the proα-chains are synthesized as larger precursors. In addition to the short N-terminal signal peptide, they also have additional amino acids, called propeptides, at both their N-and C-terminal ends (N-P and C-P in Figure 4.A). For post-translational modifications in collagen biosynthesis several specific enzymes are required. Proline and lysine residues in the Y-position are hydroxylated to 4-hydroxyproline and hydroxylysine, respectively, and some of the proline in the X-position are hydroxylated to 3-hydroxyproline. Carbohydrate moieties can be attached to some hydroxylysine and lysine residues by specific transferases. C-terminal propeptides of at least type I and III pro-collagens contain asparagines-linked high-mannose type oligosaccharide side chains. Intra- and interchain disulfide bonds are formed between cysteine residues by protein disulfide isomerase (PDI), which is a subunit of prolyl-4-hydoxylase (*59*). Pro-collagen

folding and association into a triple helix requires the involvement of molecular chaperone (*60*). After formation of the major triple helix the N-terminal propeptide will be assembled (*54*). Pro-collagens are secreted out of the cell, where they are converted to collagen by proteolytic cleavage of both the N-and C-terminal propeptide extensions. N-terminal propeptide is cleaved by the collagen type-specific N-proteinases. For maximal N-proteinase activity, all three chains of the collagen molecule must be in register. C-terminal proteinases also appear to be collagen-type specific but they does not require the intact trimer as the substrate. After removal of the propeptides the collagen monomers spontaneously assemble into fibrils by an entropy-driven process. Once the fiber is formed, the associations are stabilized by intermolecular crosslinks that provide the fiber with tremendous tensile strength and insolubility. Most of the cross-links form between the telopeptides at each end of collagen molecules. Collagen fibril formation is a complex process that is regulated by a number of different factors, including the collagen type present, the sequence and extent of propeptide processing, interactions with other matrix components such as proteoglygans, as well as a direct involvement of the cells (*61*).

Fibrillar collagens

Based on their protein and gene structures, types I, II, III, V, and XI collagens have been assigned to the fibril-forming group (*58*). They all contain a globular N-terminal domain that includes a short triple helical sequence, a major uninterrupted triple helical domain of approximately 1000 amino acids and a globular C-terminal domain. These collagens can be divided into two groups, major (I, II and III) and minor fibrillar collagens (V and XI), based on the quantities of proteins in tissue. Type II and XI collagens are found mainly in cartilaginous tissue. In connective tissues, other than cartilage, collagen fibrils are mainly composed of type I III and V collagens at different molecular rations, with diameters ranging from 20 to 500 nm (*57*). Fibrillar collagen molecules are either homotrimers with α-chains of the same kind or heterotrimers composed of two or three different α-chains. These α-chain molecules consist of an uninterrupted triple helix of approximately 300 nm in length and 1.5 nm in diameter flanked by short extra-helical telopeptides. The lateral interaction between the homologous regions within the triple helical domains is the basis for fibril formation. Fibrillar collagens self-assemble into cross-striated fibrils observed in electron microscopy in negatively stained fibrils (Fig. 2.)
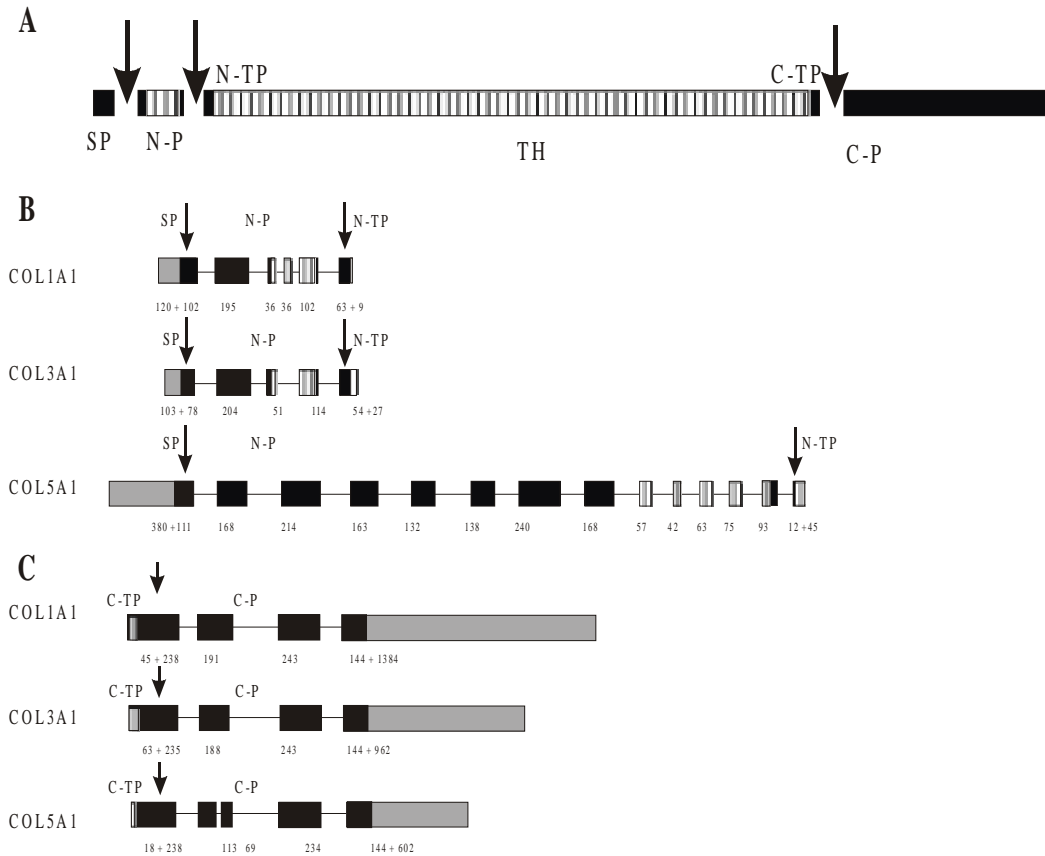
Figure 4. General domain structure of fibril-forming collagens (A) and comparison of the exon organization of the N-propeptide (B) and C-propeptide (C) of COL1A1, COL3A1 and COL5A1 genes. Exon sizes are represented in basepairs below exon boxis. Intron sizes are not proportionately represented. Arrows mark the sites of postranslational cleavage. ▯ , triple-helix coding sequences, ■ non-triple-helix coding sequences; ▨ , non-coding sequences; SP, signal peptide; N-P, N-propeptide; N-TP, N-telopeptide; TH, triple-helix; C-TP, C-telopeptide; C-P, C-propeptide. Data are collected from refereneces (*62, 63*).

The gene structure of the fibril-forming collagens

There are over 30 different collagenous polypeptides, each being a distinct gene product. With a few exceptions, the collagen genes are widely scattered on the human genome, and each gene contains its own regulatory segments, with no evidence of a singular master regulatory element (*62*). The genomic organization of the human fibrillar collagens genes has been determined (Table II). Characterization of the genomic organization of the COL1A1, COL1A2, COL3A1 and COL5A2 genes shows that these genes have very similar exon organization especially in the triple helical domain (*64, 65*). They all have 51 –52 exons and the major triple helical domain is encoded by 41-42 exons. These genes all display the exact same pattern of exon sizes in triple helical domain with one exception; the COL3A1 has an additional Gly-X-Y

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triplet in the exon 6 (*62*). All the exons for the major triple helical domain begin with a complete codon for glycine, and the number of base pairs in each exon is a multiple of 9. The N-propeptides of fibrillar collagens exhibit a much higher degree of divergence both in length and in domain structure than the rest of the polypeptide and the C-propeptides share the highest degree of sequence similarity between different types of fibrillar collagens (Fig. 4.). The collagen gene COL5A1 is one of the most interrupted genes yet described (*63*). The organization of the COL5A1 triple-helix region diverges from the fixed intron/exon organization shared by the major fibrillar collagens. However, similarities in overall structure and in coding sequences allow an unambiguous alignment of fibrillar collagen genes. The pro-α1(V) N-propeptide is encoded by 14 exons (Fig. 4.). The exon 14 is a junctional exon which is found in all of the major fibrillar genes. The N-propeptides of all minor fibrillar collagens contain a very large globular domain (*63*).

Table II Gene and protein data

GENE	Chromosome Localization	Ref.	Gene Kb	Exons	Ref.	mRNA Kb	NCBI Nucleotide Accession nr.	Amino-acid aa (ORF)	Protein kD*	NCBI Protein Accession nr.
Type I col α1	17q21.3-q22.1	*(66-68)*	17.5	51	*(69-72)*	5921	NM000088	1464	139	NP000079
Type I col α2	7q22.1	*(73)*	38	52	*(64, 74)*	5084	NM000089	1366	129	NP000080
Type III col α1	2q32.2	*(75, 76)*	39	51	*(65, 77, 78)*	5489	NM000090	1466	139	NP000081
Type V col α1	9q34.2-q34.3	*(79)*	~750	66	*(63)*	6496	NM000093	1838	184	NP000084
Type V col α2	2q31	*(75)*	67	51	*(65, 80-82)*	6217	NM000393	1496	145	NP000384
Type V col α3	19p13.2	*(83)*	51	66?	*(83)*	6200	NM015719	1745	168	NP056534
MMP2	16q21	*(84)*	27	13	*(85)*	3069	NM004530	660	74	NP004521
MMP9	20q11.2-q13.1	*(86)*	7.7	13	*(87)*	2334	NM004994	707	78	NP004985
MMP14	14q11-q12	*(88)*	11	10	*(89)*	3558	NM004995	582	66	NP004986
TIMP2	17q25	*(90)*	19	5	*(91)*	1075	NM003255	220	24	NP003246
TGF-β1	19q13.1-13.3	*(92)*	23.6	7	*(93)*	2745	NM000660	391	44	NP000651
CTGF	6q23.1	*(94)*	3.1	5	GeneBank	2312	NM001901	349	38	NP001892
PDGF A	7p22	*(95, 96)*	24	6 or 7	*(97, 98)*	2797	NM002607	211	24	NP002598
PDGF B	22q12.3-q13.1	*(99, 100)*	24	7	*(101)*	3373	NM002608	241	27	NP002599

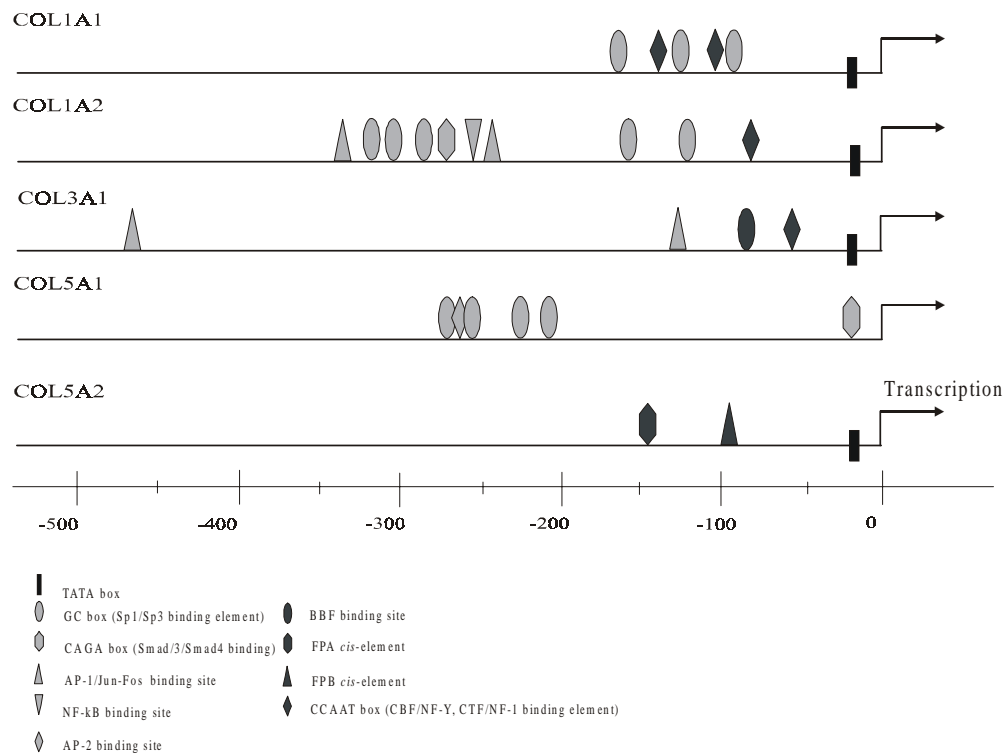| *	data collected from GeneCards database (Weizmann Institute of Science)
NCBI	National Center for Biotechnology Information
GeneBank	NCBI nucleotide database
ORF	open reading frame

Figure 5. Promoter regions of human fibrillar collagen genes. Based on following references: COL1A1 (*102, 103*), COL1A2 (*104-108*), COL3A1 (*78, 109, 110*), COL5A1 (*111*), COL5A2 (*81, 112, 113*).

Regulation of collagen genes

The mechanisms that determine both the normal tissue-specific pattern of collagen gene expression and the elevated expression in fibrosis are complex. Both transcriptional and post-transcriptional mechanisms have been described. The expression of two pro-α1(I) chains and one pro-α2(I) chain is intricately coordinated in a 2:1 ratio, and the levels of expression differ in a cell-and tissue-specific manner (*62, 114*). However, there appear to be few obvious similarities in sequence between the COL1A1 and COL1A2 promoter, except for the region around the initiation site of translation; both promoters contain characteristic TATA and CCAAT sequences (*70, 115*). Both positive and negative cis-acting regulatory elements in the human COL1A1 promoter have been found and these regulatory regions function differently in collagen-producing and non-producing cells (*103*). A number of transcription factors that bind to the *cis*-regulatory elements of the COL1A1 have been identified (*102, 116, 117*) (Fig. 5.). The DNA binding activity stimulated by TGF-β1 has been identified (*103*). Several positive

and negative elements within the first intron of COL1A1 have been identified (*118*). *Cis*-acting DNA elements that direct high and tissue-specific transcription of the human COL1A2 promoter are comprised to the area about 350 nucleotides upstream of the transcriptional initiation site (Fig. 5.). The constitutive activity of the human COL1A2 promoter is demonstrated to be regulated equivalently by the three positive *cis*-acting elements and one possible transcriptional repressor was found (*108, 119, 120*). TGF-β stimulation of human COL1A2 promoter is mediated by a multiprotein complex that interacts with two distinct promoter segments termed TGF-β-response element (TbRE). Transcription factors which bind to this complex have been identified (*105, 106*). Putative regulatory elements controlling human COL3A1 gene has been found; a TATA consensus element and two potential transcription factor binding sites (*78*). The promoter of the human COL5A1 is shown to have a number of features characteristic of the promoters of "house-keeping " and growth control-related genes in that it is GC-rich. It lacks obvious TATA and CAAT boxes and has multiple transcription start sites. A minimal promoter region of COL5A1 gene is shown to contain a number of binding sites for several transcription factors (*111*) (Fig. 5.). The shortest DNA sequence capable of directing high and cell type-specific transcription from the human COL5A2 gene include a TATA-like element and two positive regulatory sequences (*81, 113*) (Fig. 5.). Trans-acting factors binding to one of these elements are indentified and combinatorial interactions among these factors may involve in regulating tissue-specific production of type V collagen (*112*).

Post-transcriptional mechanisms involve the mRNA stability and mRNA splicing. A highly conserved sequence is found around the translation initiation site in the three collagen mRNAs, proα1(I), proα2(I) and proα1(III) (*70, 115, 121*). This region of the collagen mRNAs contains an inverted repeat sequence with the potential for forming an intramolecular 5'-stem-loop structure (*115, 122*). This region provides a potential mechanism for translational regulation. The stem-loop has been shown to decrease type I collagen mRNA stability and so to inhibit translation (*123*) and it has been suggested to be involved in the regulation of feedback translational repression by N- and C-terminal propeptides (*122*). Translational repression of pro-collagen mRNAs by N-terminal and C-terminal propeptides play significant role in the control of collagen biosynthesis. Intact N-terminal propeptide of either type I or type III pro-collagen could selectively inhibit pro-collagen biosynthesis by human fibroblasts and the C-terminal propeptide of the human α2(I) pro-collagen chain inhibits both collagen and fibronectin synthesis by human fibroblasts (*124, 125*). Stability of most mRNAs is determined

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by sequences in their 3' untranslated regions (*126*). Polymorphic RNA transcripts have been identified for the proα1(I), proα2(I), proα1(III), proα1(V), proα2(V), proα3(V) genes (*70, 74, 77, 83, 127*). A 67 kDa protein has been shown to bind to the 3' untranslated regions present in both species of collagen α1(I) mRNA, suggesting a stabilizing role of this protein (*128*). TGF-β affects the ratio and the half-life of the different forms of the proα1(I) collagen mRNA (*129*). Various cytokines and growth factors regulate the collagen genes. TGF-β increases synthesis of type I, III, and V collagen from fibroblast cell lines (*130*). The effects of interleukin 1 (IL-1) on fibroblast collagen production are controversial. IL-1 has been shown both to increase and inhibit collagen production (*131, 132*). Tumor necrosis factor α (TNF-α) inhibits collagen production *in vivo* and *in vitro* (*50, 133-135*). Interferon γ (IFN-γ) inhibits the transcription of collagen in fibroblasts and abrogates its stimulation induced by TGF-β (*136, 137*). Antagonistic activity against TGF-β effect on the COL1A1 by IL-1 has been observed at the mRNA level in human skin fibroblasts and on the COL1A2 by TNF-α (*107, 138*). The combination of TNF-α and IFN strongly reduces of the collagen mRNA levels indicating that the two cytokines act synergistically (*136*). Epidermal growth factor (EGF) can down-regulate type I and III pro-collagen mRNA levels, in addition to its action in fibroblast proliferation (*49*). Studies in response of different platelet-derived growth factor (PDGF) isomers on collagen mRNA expression have yielded variable results. In lung fibroblasts steady-state levels of pro-collagen α1(I) and pro-collagen α1(III) mRNA were not changed after exposure to any PDGF isoforms (*139*). On the other hand in human fibroblasts cultured from normal wounds, PDGF-AA and PDGF-BB down-regulated both the steady-state level of proα1(I) and α1(III) collagen chain mRNAs, whereas PDGF-AB in low concentrations up-regulated and in high concentrations down-regulated the expression of type I and III pro-collagen mRNAs (*140*). Other factors such as various hormones and vitamins have also an ability to regulate collagen synthesis (Table III) (*141-145*).

Table III Hormones and vitamins which modulate the type I collagen

Modulators	Type I collagen synthesis	References
Ascorbic acid	↑	(*146*)
Glucocorticoids	↓	(*142*)
Prostagladin E$_2$	↓	(*143*)
Retinoic acid	↓	(*144*)
Vitamin D	↓	(*145*)

Type I collagen

Type I collagen represents the prototype of the fibrillar collagens. It is the major collagen in most tissues. Many of the other fibril-forming collagens have a more selective tissue distribution (Table I). Type I collagen is the predominant collagen component of bone and tendon and is found in large amounts in skin, aorta, and lung (*147*). Type I collagen fibers provide great tensile strength and limited extensibility. The most abundant molecular form of type I collagen is a heterotrimer composed of two different α-chains $[\alpha 1(I)]_2 \alpha 2(I)$. Type I collagen can directly promote the adhesion and migration of numerous cell types, including hepatocytes, keratinocytes and fibroblasts (*148-150*). In wound healing type I collagen gene expression is found in every phase of repair process (*151, 152*). Its synthesis coincides with increased wound-breaking strength (*47*). Ultimately in wound healing, the rather acellular but fiber-rich scar tissue contains predominantly fibrils derived from type I collagen molecules (*147*). Type I collagen thus gradually replaces the other collagen types when the wound matures to scar.

Type III collagen

Type III collagen molecule is a homotrimer of three identical α-chains $[\alpha 1(III)]_3$. It is widely distributed in soft connective tissues, and in most tissue is co-expressed with type I collagen, the major exception being bone matrix, which does not contain any of type III collagen (*153*). The ratio of the two collagen types varies considerably in different tissue, during development and granulation tissue formation, and in some disease processes (*154-157*). Higher proportions of type III collagen are usually found in distensible connective tissues such as blood vessels (*158*). Due to its abundance in fetal tissue type III collagen has also been called fetal or embryonic collagen (*154, 155*). The ratio of type III to type I collagen increases in the early stage of skin wound healing (*156, 159*). The proportion of type III collagen out of the total collagen contents is about 20% and 50% in adult human skin and embryonic dermis,

respectively (*160*). Liver has as much as 45% and human lung 21% of type III collagen (*161, 162*). Takasako et al. (*163*) reported the total quantitity of type III collagen to decrease upon aging in all tissues. In the mature organism elevated amounts of type III collagen have been detected during repair processes, e.g., in healing skin wounds and tendons, and in experimental granulation tissue (*156, 159, 164*). The chains of type III collagen, unlike those of other fibrillar collagens, are connected by intramolecular disulfide cross-links that occur within the triple helical portion of the molecule (*153*). Other unique features of type III collagen are; high levels of 4-hydroxyproline and glycine and the presence of half-cystines, which generate the intramolecular disulfide cross-links. It has been suggested that the extra glycine residues may cause localized helix instability, resulting in increased susceptibility to proteolytic cleavage and more rapid turnover of matrix containing this collagen type (*153*). The mechanisms regulating the change in the ratio of type I and III collagens are poorly understood. In cultured cells the ratio of type I and III collagen synthesis usually parallels the ratio of the corresponding mRNAs which suggest a coordinated control at the transcriptional level (*165*).

Type V collagen

Type V collagen is a quantitatively minor fibrillar collagen with a broad tissue distribution. It was first isolated from human placenta (*166*). It is expressed in many connective tissues, blood vessel walls, and the kidney (*167*). Three different chains $\alpha 1(V)$, $\alpha 2(V)$, and $\alpha 3(V)$ encoded by three different genes participate in the formation of the type V collagen molecule. The quantitatively major triple-helical assembly of type V collagen is the $[\alpha 1(V)]_2\, \alpha 2(V)$ molecule, found in the skin, bone, cornea and placenta (*61, 166, 168, 169*). An $[\alpha 1(V)]_3$ homotrimer in cell cultures as well as an $[\alpha 1(V)\alpha 2(V)\alpha 3(V)]$ form in human placenta have also been reported (*170, 171*).

Unlike the major interstitial collagens, the tissue form of pro-collagen V does not undergo complete processing despite the presence of putative N-proteinase and C-proteinase cleavage sites in the reported cDNA-derived amino acid sequences of $\alpha 1(V)$ and $\alpha 2(V)$ (*170, 172-174*) (Fig. 6.). The greatest variability between fibrillar collagen structures lies in their N-terminal propeptides, which may reflect diversity in the functional roles of these molecules. The amino acid sequence of this N-terminal region of the $\alpha 1(V)$ molecule predicts several domains. Co-polymerisation of small amounts of type V collagen with the major fibrillar collagen type I can limit fibril diameters due to steric hindrance (*175*). The $\alpha_2(V)$ N-propeptide is only partially processed following secretion of $[\alpha 1(V)\,]_2\, \alpha 2(V)$ heterotrimers from cells; incorporation of
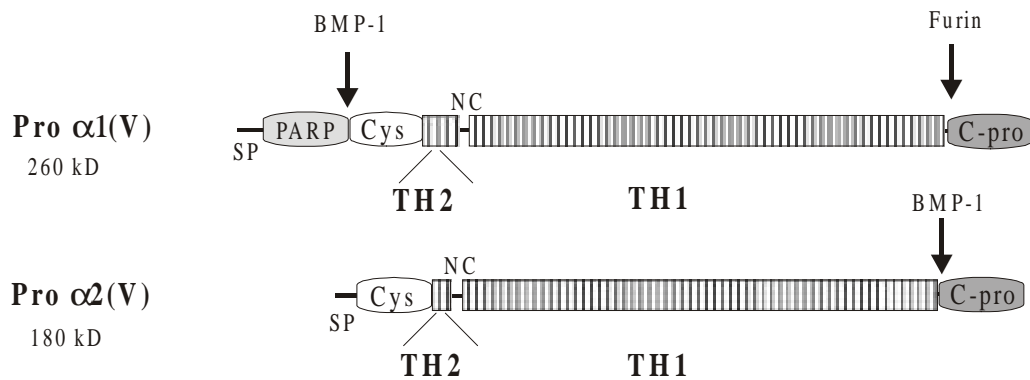
Figure 6. Type V collagen protein structure. Human pro-α3(V) collagen chain has the same overall N-terminal structure with PARP domain as pro-α1 chain (*83*). TH = triple helix, Cys = cysteine-rich domain, PARP = proline and arginine rich peptide, SP = signal peptide, NC = non collagen. Modified from (Unsöld et al., 2002) (*174*).

such heterotrimers onto the surface of type I collagen containing fibrils would allow then α-propeptide to project from the surface of the fibrils (*176*). Within type I/V heterotypic collagen fibrils, the entire of the triple-helical domain of each type V collagen molecule lies within a shell of type I collagen molecules (*167*). Type V collagen chains also form heterotypic molecules with type XI collagen chains (*177*).

An anchoring function between basement membranes and stromal matrix has been proposed for type V collagen based on the localization of type V collagen as thin fibrils between the basement membrane and the matrix (*178, 179*). An interaction between cells and type V collagen was observed by the requirement of type V collagen synthesis for epithelial cell migration (*180*). Smooth muscle cells preferentially bind to type V collagen while endothelial cells only transiently attach to type V collagen (*181, 182*). The adhesion and anchoring can happen by both Arg-Asp-Gly (RDG) sequence-dependent and –independent manner, depending on the mediating integrins (*183, 184*). Type V collagen can also be anti-adhesive and inhibit cell attachment to fibronectin (*183, 185*). In mature tissues type V collagen epitopes are probably masked and difficult to detect due to the incorporation of type V collagen molecules in type I collagen fibrils (*175*).

The susceptibility of type V collagen for degradation by matrix metalloproteinases (MMP) differ from that of collagen types I, II, and III. Both MMP-2 and MMP-9 can cleave the triple-helical domain of type V collagen but not those of type I collagen (*186*). Type V collagen is also susceptible to trypsin and thrombin digestion (*169, 187*). The resistance of type V collagen to collagenase digestion that can occur during inflammation can prevent its rapid degradation

in the matrix as compared to type I collagen (*167*). Triple-helical type V collagen trimers bind to heparin with different affinity, $\alpha 1(V)_3$ homotrimer binds most strongly (*188*). In addition to heparin, type V collagen interacts with the other matrix proteoglycans such as the two small proteoglycans decorin and biglycan (*189*). When bound to type V collagen, both decorin and biglycan accelerated the inhibition reaction of heparin cofactor II-thrombin on plasma serine proteinase. Following endothelial injury, the collagen-proteoglycan complex, which is likely exposed at the surface of blood vessels, acts as a "thrombo-resistant surface". The triple-helical domain of $\alpha 1(V)$ collagen binds specifically to a variety of other molecules, such as thrombospondin, insulin, fibronectin and even DNA (*185, 190-192*). Type V collagen has been identified in vivo in vascular subendothelia and on the endothelial cell surface (*193, 194*). Type V collagen is a poor adhesive substrate for platelets in vitro. Under flow conditions platelets are non-adhesive to type V collagen (*195, 196*). The thrombo-resistance of the endothelium has been attributed to the presence of type V collagen on the luminal surface of the endothelium (*193*). The localization of type V collagen in capillary basement membranes and in the subendothelium of large vessels is consistent with its potential role as an early-synthesized component of the matrix upon which cells migrate (*193, 197, 198*).

Cell and ECM interaction
Extracellular matrix (ECM) provides the physical microenvironment in which cells live; it provides a substrate for cell anchorage, serves as a tissue scaffold and guides cell migration during wound repair. A tight balance between ECM synthesis and breakdown is required for the normal functioning of all tissues. The amount and composition of ECM are controlled by growth factors and the mechanical stress acting on a tissue (*199, 200*). In addition to growth factor signaling mechanisms inside the cell,signaling can also be regulated outside the cell by extracellular matrix proteins and proteolytic enzymes (*201*). Many growth factors have been found to be associated with the extracellular matrix proteins or with heparan sulfate. Rapid and localized changes in the activity of these factors can be induced by the release from matrix storage and/or by activation of latent forms. These growth factors, in turn, control cell proliferation, differentiation, and synthesis and remodeling of the extracellular matrix (*202*).
The communication between collagens and cells is achieved by cell surface receptors. Three types of cell surface receptors for collagen are known: integrins, discoidin domain receptors and glycoprotein VI (*203*). All three receptor types independently trigger a variety of signaling pathways upon collagen-binding. Besides regulating numerous cellular responses, both integrin

and discoidin domain receptors monitor the integrity of the collagenous extracellular matrix by triggering matrix degradation and renewal (*203*). Two of the best known collagen receptors are members of the integrin family, α1β1 and α2β1 (*204*). The α1β1 integrin is abundant on smooth muscle cells, whereas α2β1 is the collagen receptor on platelets and epithelial cells. Many cell types, including fibroblasts, chondrocytes, osteoblast, endothelial cells, and lymphocytes may express both of the receptors simultaneously. The integrins are connected to cellular signaling pathways. The shape of the matrix and ultimately the shape of the cell can modify signaling events (*204-206*).

Cell movement, occurring during tissue repair, depends on integrin-mediated interactions (*207*). Integrins physically link the ECM to the cytoskeleton, and hence are responsible for establishing a mechanical continuum by which forces are transmitted between the outside and the inside of cells in both directions (*208*). Fibroblasts embedded in a restrained collagen lattice transmit mechanical forces by integrin receptors (*200*). This interaction results in the induction of growth factors including TGF-βs and CTGF and in enhanced collagen production. Simultaneously, the expression of MMP-1 and MT1-MMP is down-regulated, resulting in an overall ECM synthesis favoring phenotype (*200*). During wound healing process α1β1 integrin expression is down-regulated and α2β1 integrin expression is up-regulated in fibroblasts. This is due to the action of PDGF and TGF-β (*32, 209*).

Myofibroblasts are a particular phenotype of granulation tissue fibroblasts which show an abundant rough endoplasmic reticulum and usually express α-smooth muscle (α-SM) actin (*210*). Morphologically, myofibroblasts are characterized by a contractile apparatus that contains bundles of actin microfilaments with associated contractile proteins such as non-muscle myosin, and which is analogous to stress fibers that have been described in cultured fibroblasts (*211*). These actin bundles terminate at the myofibroblast surface in the fibronexus – a specialized adhesion complex that uses transmembrane integrins to link intracellular actin with extracellular fibronectin fibrils, a phenomenon not found in normal fibroblasts (*211, 212*). Functionally, this provides a system where the force generated by stress fibers can be transmitted to the surrounding ECM (*211*). In addition, extracellular mechanical signals can be transduced into intracellular signals through this system (*199, 211*). There are two types of myofibroblasts: those that do not express α-SM actin, which is termed "proto-myofibroblasts"; and those that do express α-smooth muscle actin, which is termed "differentiated myofibroblasts" (*212*). In normal tissues, proto-myofibroblasts are always present when there

is the need to generate mechanical tension. PDGF is important in the formation of the proto-myofibroblast during wound healing but does not induce the expression of α-SM actin or the formation of the differentiated myofibroblasts (*1*). TGF-β1 has a key role in stimulating the proto-myofibroblasts to differentiate (*213*). The expression of α-smooth muscle actin and collagen type I in these cells is coordinately regulated by TGF-β1 (*30*). Thus, the cell-ECM interaction modulates the phenotype of fibroblasts as the wound repair progresses.

DEGRADATION OF EXTRACELLULAR MATRIX

Matrix metalloproteinases
Degradation of extracellular matrix (ECM) proteins is essential in many physiological processes, e.g., during development, growth, and tissue repair. On the other hand, excessive proteolysis plays an important role in numerous pathological conditions, such as rheumatoid arthritis, osteoarthritis, disorders of skin, and periodontitis (*214-216*). Proteolytic enzymes are classified as either exopeptidases or endopeptidases based on whether they cleave terminal or internal peptide bonds, respectively. Endopeptidases are classified as serine, cysteine, aspartic or metalloproteinases based on amino acid sequences and cofactors determining their catalytic activity and mechanism. Matrix metalloproteinases (MMPs) form one of the four subfamilies that belong to metzincins, which in turn is one of numerous metalloproteinase superfamily (*217*). The first member of this family was found attacking triple-helical collagen in resorbing tadpole tails in metamorphosis (*218*). MMPs play important roles in wound healing, angiogenesis, embryogenesis and in pathological processes such as tumor invasion and metastasis (*219*). In addition to the proteolytic degradation of ECM molecules, recent data has extended the substrate specificity of MMPs to include enzyme inhibitors, such as α1-proteinase inhibitor, cell-bound precursors of cytokines and active cytokines, such as TNF-α and IL-1β , and adhesion molecules, such as L-selectin (*220-223*).

Currently the MMP family consists of 25 distinct but structurally related vertebrate enzymes and 21 characterized human homologues (*217*). They are zinc-dependent neutral endopeptidases. Depending on substrate specificity, amino acid similarity and identifiable sequence modules, the family of MMPs can be classified into the following distinct subclasses: collagenases, gelatinases, stromelysins, matrilysins and membrane-type matrix metalloproteinases (MT-MMP) (Table II and IV) (*224*).

Table IV Matrix metalloproteinases

MMP	Enzyme name(s)	Potential substrates	
		Matrix components	Others
Collagenases			
MMP-1	collagenase-1 fibroblast collagenase, interstital collagenase	Col I, II, III, VII, VIII, X, XI; gelatin; entactin; aggrecan; tenascin; MBP; perlecan; IGFBP-2,3	ProMMP-1,2; casein, α2M; α1PI; α2AC; proTNFα
MMP-8	collagenase-2 neutrophil collagenase	Col I, II, III; gelatin; entactin; aggrecan; tenascin	ProMMP-8; α2M; α1PI
MMP-13	collagenase-3	Col I, II, III, IV, IX, X, XIV; gelatin; entactin; aggrecan; tenascin; osteonectin; fibrinogen/fibrin	ProMM-9,13; α2M; α2AC; PAI
Gelatinases			
MMP-2	gelatinase A 72-kDa gelatinase	Gelatins; fibronectin; elastin; Col I, IV, V, VII, X, XI; laminin; aggrecan; vitronectin; decorin; MBP; IGFBP-3/5	ProMMP-1,2,13; plasminogen; casein; α2M; α2AC; proTNFα; proTGFβ2; proIL1β; MCP3; FGFr1
MMP-9	gelatinase B 92-kDa gelatinase	Gelatins; Col IV, V,VII, XI, XIV, XVII; elastin; fibrillin; aggrecan; fibronectin; fibrinogen/fibrin; MBP	Plasminogen; casein; α2M; α1PI; proTNFα; proTGFβ2; proIL1β
Stromelysins, matrilysins and others			
MMP-3	stromelysin-1	Fibronectin; laminin; gelatins; Col III, IV, V, VII, IX, X, XI; decorin elastin; nidogen; perlecan; entactin; aggrecan; vitronectin; tenascin; fibrin/fibrinogen; fibrillin; IGFBP-3	ProMMP-1,3,7,8,13; plasminogen; casein; α2M; α1PI; α2AC; proTNFα; E-cadherin; proIL1β; proIL-1β; proHB-EGF
MMP-10	stromelysin-2	Fibronectin; laminin; gelatins ; Col III, IV, V, II, IX, X,XI; decorin; elastin, nidogen; fibrin/fibrinogen; fibrillin;entactin; tenascin; vitronectin; aggrecan	ProMMP-1,8,10
MMP-11	stromelysin-3	Laminin; fibronectin; aggrecan; IGFBP-1	α2M; α1PI
MMP-7	matrilysin-1 (PUMP-1)	Fibronctin; laminin ; Col IV; gelatins; elastin: aggrecan; decorin; nidogen; fibrillin; laminin; MBP; osteonectin; tenascin; vitronectin	ProMMP-2,7; casein; α1PI; pro α-defensin; FasL; β4 integrin; E-cadherin; plasminogen; proTNFα
MMP-26	matrilysin-2 (endometase)	Col IV; gelatin; fibronectin; fibrin/fibrinogen	ProMMP-9; casein; α1PI
MMP-12	macrophage metalloelastase	Elastin; fibronectin; fibrinogen/fibrin; laminin	Plasminogen; casein
MMP-19	RASI	Col IV; gelatin; fibronectin; tenscin; aggrecan; COMP	
MMP-20	enamelysin	Amelogenin; aggrecan; COMP	
MMP-23	CA-MMP	Gelatin	
MMP-28	epilysin	ND	Casein

Modified from (McCawley et al, 2001 and Sternlicht et al, 2001)(*217, 225*). Abbreviations: Col, collagen; COMP, cartilage oligomeric matrix protein; IGFBP, insulin-like growth factor binding protein; Ln, laminin; MBP, myelin basic protein; PAI, plasminogen activator inhibitor; α2M, α2 macroglobulin; α1PI, α1 proteinase inhibitor; α2AC, α2 antichymotrypsin; ND, not determined.

The MMPs are organized into structural domains that impart their specific biological functions (Fig. 7.). All members of the family share the propeptide domain that is lost upon activation,

and the catalytic domain, which contains a zinc-binding site (*224*). The hinge-region marks the transition to the C-terminal domain. The hemopexin- or vitronectin-like C-terminal domain is likely to play a role in encoding substrate specificity and is present in all MMPs except in matrilysins and MMP-23 (*217, 224*). The gelatinases possess an insert within the catalytic domain that provides the enzymes with gelatin-binding properties (*226*). The transmembrane domain of the membrane-type MMPs target their distribution to the cell surface.

Gelatinases

MMP-2 (gelatinase A, 72-kDa gelatinase) and MMP-9 (gelatinase B, 92-kDa) differ from the other MMPs by containing three head-to-tail repeats homologous to the type II repeat of the collagen binding domain of fibronectin. These domains are required for gelatinases to bind and cleave collagen and elastin (*226, 227*). Similarly to collagenases, the hemopexin domain of MMP-2 is critical for the initial cleavage of the triple helical type I collagen (*228*). In addition, MMP-9 has a unique type V collagen-like insert of unknown importance at the end of its hinge region.

MMP-2 is produced constitutively *in vitro* by most cells of fibroblastic, endothelial, and epithelial origin (*224*). Expression of MMP-9 is more restricted and is often low in normal tissues, but can be induced when tissue remodeling occurs during development, wound healing and cancer invasion. It is actively expressed by polymorphonuclear leukocytes, macrophages, epithelial-derived cells and osteoclasts (*229-231*) (*232*). While both gelatinases can degrade a variety of proteins *in vitro*, the *in vivo* substrates are largely unknown. Both MMP-2 and MMP-9 efficiently degrade denatured collagens (i.e. gelatins) of all genetic types, and these enzymes also attack basement membranes, fibronectin, and insoluble elastin. Unlike collagenases 1 and 2, gelatinases are capable of degrading type IV and V collagens. In addition, MMP-2 has been reported to degrade also native type I collagen and to cleave MMP-9 to its active forms (*233, 234*). The expression of both gelatinases correlates with invasive potential of various tumors (*219*). MMP-9 has been suggested to affect angiogenesis by releasing ECM bound vascular endothelial growth factor (VEGF) (*235*).

Other MMPs

Collagenases -1, -2, and -3 (MMP-1, MMP-8, and MMP-13, respectively) are the principal secreted neutral proteinases capable of initiating the degradation of native helix of fibrillar collagens of type I, II, and III (*224*). The hemopexin domains of these MMPs are essential for
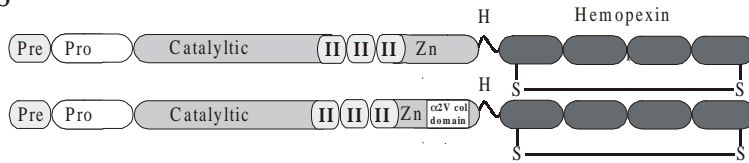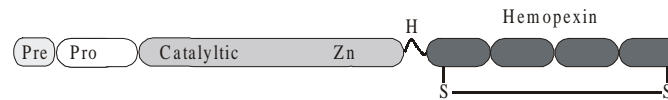
Collagenases

MMP-1
MMP-8
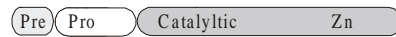MMP-13
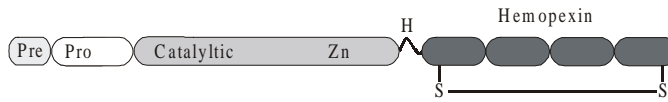
Gelatinases

MMP-2
MMP-9

Stromelysins and matrilysins
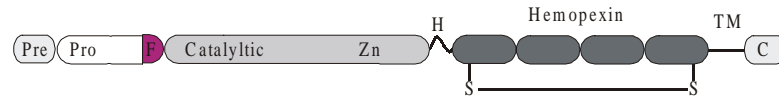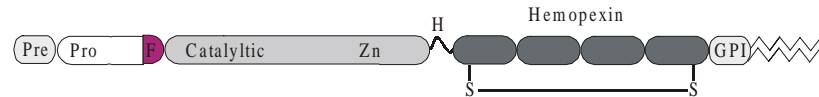
MMP-3
MMP-10
MMP-12

MMP-7
MMP-26

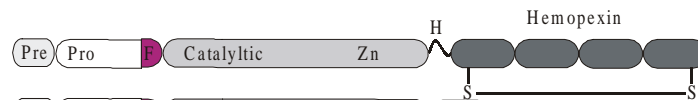# Membrane-type MMPs

MMP-14
MMP-15
MMP-16
MMP-24

MMP-17
MMP-25

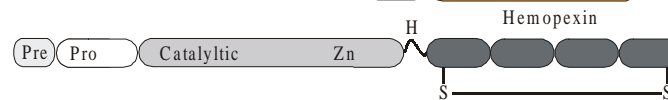# Other MMPs

MMP-11
MMP-28

MMP-23

MMP-19
MMP-20

Figure 7. Domain structure of the MMPs. Pre, signal sequence; Pro, propeptide; F, furin-susceptible site; Zn, zinc-binding site; II, collagen-binding fibronectin type II inserts; H, hinge region; TM, transmembrane domain; C, cytoplasmic tail; GPI, glycophosphatidyl inositol-anchoring domain; C/P, cysteine/proline; IL-1R, interleukin-1 receptor; α2V col, α2V collagen domain. The hemopexin/vitronectin-like domain contains four repeats with the first and last linked by a disulfide bond. Modified from (Sternlicht et al, 2001 and Cawston et al, 1998) (*217, 236*).

specific binding and cleavage of the substrate (*228, 237*). The fibrillar collagens are cleaved at a specific site to yield N-terminal ¾ and C-terminal ¼ fragments, which denature to gelatin and can be further degraded by other MMPs, e.g., gelatinases (*36, 224*). MMP-1 degrades preferentially type III collagen, while MMP-8 has preference for monomeric types I and II collagens (*238, 239*). MMP-13 is ten-fold more effective in degrading type II collagen, has a stronger gelatinolytic activity than collagense-1 and a broader substrate specificity than the other collagenases (*237*).

The initial cleavage of native collagen by collagenases represents the rate-limiting step in the degradation of interstitial collagens (*238*). MMP-1 is expressed in various normal cell types such as fibroblasts, keratinocytes, endothelia cells, monocytes, macrophages, chondrocytes and osteoblasts (*224*). MMP-8 is synthesized by polymorphonuclear leukocytes during their maturation in bone marrow, stored in intracellular granules, and released in response to external stimuli (*240*). In addition, fibroblasts, bronchial epithelial cells and macrophages express MMP-8 (*241, 242*). Both MMP-1 and MMP-8 are present at high levels during the inflammatory and early proliferative phases of wound repair (*243*). MMP-13 is expressed during fetal bone development, postnatal bone remodeling, and gingival wound repair (*244, 245*).

The stromelysin subgroup contains stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and two other MMPs with similar substrate specificities that are structurally less closely related: namely MMP-7 and macrophage metalloelastase (MMP-12). An important feature of this group of enzymes is their capacity to activate pro-collagenases. MMP-3 and MMP-10 are expressed by fibroblastic cells and by normal and transformed squamous epithelial cells (*246, 247*). Stromelysins degrade basement membrane components, type IV collagen nidogen, and fibronectin; both matrilysin and macrophage metalloelastase have the ability to degrade elastin (*36, 224*).

The existence of membrane-bound MMPs was suggested by the finding that plasma membranes from various tumor cells contained proMMP-2 activator sensitive to MMP inhibitors (*248, 249*). This lead to the identification of MMP-14 (MT1-MMP), with a transmembrane domain that directs a cell surface localization (*250*). MMP-14 is a type I transmembrane protein. It has a typical five-domain modular structure resembling collagenases and sromelysins. It also contains a domain susceptible to intracellular proteolytic activation by furin, an additional short carboxyl-terminal transmembrane domain, and intracellular domain

(Fig. 7.) (*251*). An active MT1-MMP serves as a cell membrane receptor for the complex formed of latent MMP-2 (proMMP-2) and tissue inhibitor of metalloproteinases-2 (TIMP-2) (*252*). MT1-MMP is more active in ECM degradation and promoting cell invasiveness in experimental models than its soluble form or the secretory MMPs, highlighting the importance of the cell surface localization and cellular regulation of these enzymes (*253, 254*).

Regulation of MMP activity

MMP activity can be regulated at three levels: transcription, pro-enzyme activation, and specific inhibition by TIMPS. In the adult tissues, low levels of MMP expression mediate normal matrix remodeling, while during inflammation and injury, large amounts of MMPs are produced, presumably to repair damaged ECM (*224, 255*). Most MMP genes are closely regulated at the level of transcription, with the notable exception of MMP-2, which is often
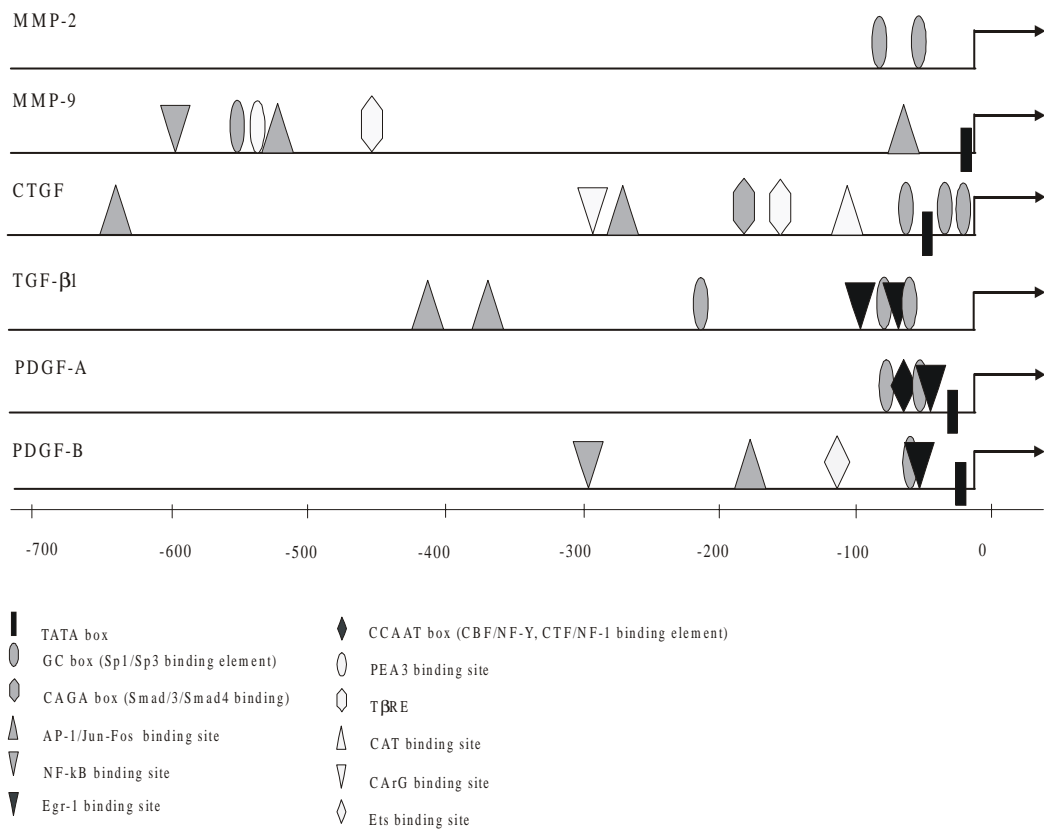

Figure 8. Promoter regions of human genes for MMP-2, -9, CTGF, TGF-β1, PDGF -A and -B. Based on following references PDGF-A (*256-258*), PDGF-B (*256, 257*), TGF-β1 (*257, 259, 260*), CTGF (*261, 262*), MMP-2 (*263*) and MMP-9 (*264, 265*).

constitutively expressed and controlled through a unique mechanism of enzyme activation (*266*). Unlike other metalloproteinases, MMP-2 is not, in general, regulated by cytokines, growth factors or hormones, with the exception of TGF-β (*267*). In addition, MMP-8 and MMP-9 stored in the secretory granules of neutrophils make an exception to the transcriptional control, since neutrophils are synthesized in bone marrow and the regulation is mediated by granule release (*240, 268*). The promoter of the MMP-2 gene does not have a TATA sequence but contains two Sp-1 sites that are important for basal promoter activity (Fig. 8.) (*85*). MMP-9 promoter contains two AP-1 sites and its expression is subject to modification by a variety of physiological signals (*87*). MMP9 gene promoter studies have identified the regions responsible for its cell-specific expression *in vivo* (*229*). The MMP14 promoter has Sp1 binding site but lacks a conserved TATA sequence and AP-1 binding site. MMP14 is constitutively expressed *in vitro* by many different cell types (*269*). MMP14 expression is regulated by cytoskeleton-ECM interactions (*270*). Increased binding of Egr-1 transcription factor to MMP14 promoter correlates with enhanced MMP14 transcription in endothelial cells cultured in collagen matrix (*271*). The induction of transcriptions factor Egr-1 occurs in response to potential angiogenesis initiators such as wound formation, mechanical stress, and fluid shear stress (*257*). The expression of MMP14 during inflammation is regulated by cytokines. TNF-α and type I collagen synergistically induces MMP14 expression in skin fibroblasts (*272*). TNF-α, IL-1α, and IL-1β also up regulate MMP14 gene expression in vascular endothelial cells (*273*). TIMPs have been thought to be mainly regulated at the level of gene expression. Various cultured cells constitutively express TIMP-2, whereas several factors/cytokines and chemicals up-regulate the expression of TIMP-1 (*274*).

Most MMPs are first synthesized as inactive pro-enzymes or zymogens. The inactive state of the enzyme is maintained by a bond between an unpaired cysteine in the prodomain and the zinc atom in the catalytic domain. Following opening of the cysteine-zinc bond, a series of autocatalytic cleavages result in excision of the remainder of the prodomain to yield a catalytically competent enzyme (*275*). This extracellular activation can be initiated by other already activated MMPs or by several serine proteinases (*217*). In addition, MT1-MMP contains a furin-susceptible site in the prodomain, which allows it to be activated prior to secretion by Golgi-associated furin-like proteases (*228*). MT1-MMP and TIMP-2 are required for the activation of latent MMP-2 (*266, 276, 277*). On the cell surface, MT1-MMP, TIMP-2 and pro MMP-2 form a ternary complex. It has been suggested that TIMP-2 combines with MT1-MMP to form a receptor for the latent MMP-2 and that free MT1-MMP may then

activate the latent MMP-2 by proteolysis. Excess TIMP-2 interferes with this activation mechanism by binding and inhibiting all available MT1-MMP molecules (*252*).

The major physiologic inhibitors of the MMPs are the family of specific tissue inhibitor of MMP (TIMP), and α-2 macroglobulin, which may be important in controlling overall proteolytic activity (*224*). In addition, thrombospondins can inhibit MMP-2 and 9 activation and induce their clearance through scavenger receptor-mediated endocytosis (*278*). The TIMP family comprises at present four structurally related members, TIMP-1, 2, and 3, and 4. They are composed of N-and C-terminal domains, which both are stabilized by three disulfide bonds between six conserved cysteine residues. The larger N-terminal domain is important for MMP inhibition. All TIMPs can inhibit most MMPs by tight non-covalent binding to their active site in a 1:1 molar ratio resulting in loss of proteolytic activity (*274*). A unique characteristic of MMP-2 and MMP-9 is the ability of their zymogens to form tight non-covalent and stable complexes with TIMPs. It has been shown that pro-MMP-2 binds TIMP-2 and pro-MMP-9 binds TIMP-1 (*279, 280*). This interaction has been suggested to provide an extra level of regulation by potentially preventing activation. Both TIMP-1 and -2 have mitogenic activities on a number of cell types, whereas overexpression of these inhibitors reduces tumor cell growth. TIMP-1 and -2 are secreted in soluble form, whereas TIMP-3 is associated with the ECM (*274*).

The role of MMPs and TIMPs in wound healing

The degradation of extracellular matrix is required to remove damaged tissue and provisional matrixes and to permit vessel formation and cell migration during wound healing. These remodeling processes involve the action of extracellular proteinases (*36*). MMP-1 is present in the wound environment and it is produced by fibroblasts, macrophages, and other cells within the granulation tissue (*281*). Basal keratinocytes at the migrating front of re-epithelialization are the predominant source of MMP-1 during active wound repair (*282, 283*). Keratinocytes seems to be a major participant in the degradation of extracellular matrix during wound healing and fibroblasts. Macrophages, and other cells within the dermis release MMP-1 only at certain stages of repair (*36*). MMP-2 and MMP-9 may be important in detaching keratinocytes from the basement membrane prior to lateral movement at the beginning of epithelial wound healing, and both MMP-2 and MMP-9 are transiently seen in epidermal cells shortly after wounding (*284, 285*). In chronic wounds, however, these gelatinases are not actively synthesized by epidermal cells and are only occasionally expressed by either resident dermal or

inflammatory cells (*283*). MMP-9 may be secreted by certain inflammatory cells that migrate to wound sites, notably neutrophils, eosinophils, and macrophages (*230, 240*). Many metalloproteinase genes are highly expressed during skin wound healing. From these, MMP-3, MMP-9 and MMP-13 were expressed early in the repair process and were found predominantly in migrating epithelial cells while those corresponding to the late expressing genes, MMP-14, MMP-9 and MMP-11 were specifically detected in wound stromal cells (*286*). These results also suggested that during cutaneous wound healing pro-MMP-2 and pro-MMP-9 are activated by MMP-14 and MMP-3, respectively. On the other hand, TIMP-1 expression was seen at the epithelial/mesenchymal border during dermal wound healing (*46*).

GROWTH FACTORS IN WOUND HEALING

Growth factors are essential for regulating the molecular and cellular events involved in the formation of granulation tissue and in wound healing. Peptide growth factors regulate many of these processes. Growth factors such as insulin-like growth factor (IGF), and fibroblast growth factors (FGF) epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF) are thought to be involved in wound healing (Table V) (*287, 288*). Different growth factors affect the different phases in wound healing differently. Growth factors induce the synthesis of themselves in positive autocrine feedback loops, as well as of other growth factors. Thus, a plethora of growth factors is likely to be present at the site of the wound, which assures efficient enhancement of the different phases of wound healing. The most important granulation modulating growth factors appear to be PDGF, TGF-β1 and CTGF (*10, 287, 289*).

TGF-β

Transforming growth factor-β (TGF-β) was first identified in vitro as a soluble factor which was capable of inducing a transformed cell phenotype (*290*). TGF-β stimulates the proliferation of connective tissue cells, but acts as a growth inhibitor of many other cell types, including epithelial and endothelial cells (*17*). It induces the synthesis of extracellular-matrix proteins, modulates the expression of matrix proteases and protease inhibitors, increases integrin expression and thus enhances cell adhesion (*6, 291, 292*). TGF- β affects also mesenchymal differentiation and is a very potent chemotactic agent for several cell types,

especially monocytes and fibroblasts (*6*). In addition to the three mammalian TGF-β isomers (TGF-β1, -β2 and -β3), the TGF-β superfamily comprises the activins, and bone morphogenetic proteins, as well as many other factors that are all thought to play major roles in differentiation and tissue morphogenesis (*293*).

Table V Growth factor signals at the wound site. Modified from (Martin, 1997)

| Growth factor	Source	Primary target cells and effect	Refs.
EGF	Platelets	Keratinocyte motogen and mitogen	(*3*)
TGF-α	Macrophages; keratinocytes	Keratinocyte motogen and mitogen	(*3, 8*)
HB-EGF	Macrophages	Keratinocyte and fibroblast mitogen	(*15*)
FGFs 1, 2, and 4	Macrophages and damaged endothelial cells	Angiogenic and fibroblast mitogen	(*4*)
FGF7 (KGF)	Dermal fibroblasts	Keratinocyte motogen and mitogen	(*4, 294*)
PDGF	Platelets; macrophages; keratinocytes	Chemotactic for macrophages, fibroblasts; macrophage activation, fibroblast mitogen, and matrix production	(*5*)
IGF-1	Plasma; platelets	Endothelial cell and fibroblast mitogen	(*8, 295*)
VEGF	Keratinocytes; macrophages	Angiogenesis	(*19*)
TGF-β1 and -β2	Platelets; macrophages	Keratinocyte migration; chemotactic for macrophages and fibroblasts; fibroblast matrix synthesis and remodeling	(*6*)
TGF-β3	Macrophages	Antiscarring	(*6*)
CTGF	Fibroblasts; endothelia	Fibroblasts; downstream of TGF-1	(*287*)
Activin	Fibroblasts; keratinocytes	Currently unknown	(*296*)
IL-1α and -β	Neutrophils	Early activators of growth factor expression in macrophages, keratinocytes, and fibroblasts	(*13*)
TNF-α	Neutrophils	Similar to the IL-1s	(*13*)
HGF	Fibroblasts	Epidermal cell proliferation and migration	(*16*)
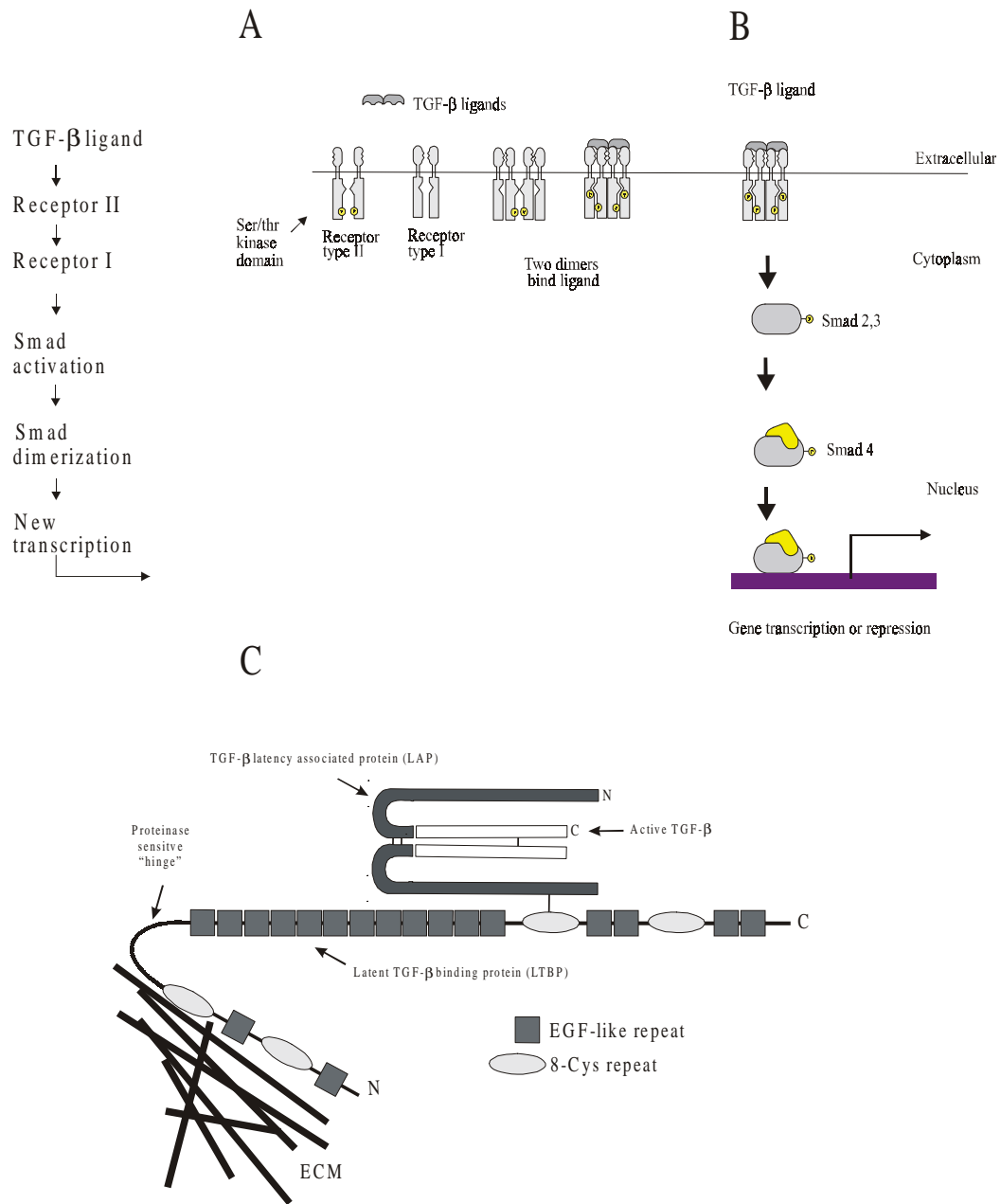
Figure 9. The Smad pathway activated by TGF-β superfamily ligands. (A) An activation complex is formed by the binding of the ligand by the type I and type II receptors. This allows the type II receptor to phosphorylate the type I receptor on particular serine or threonine residues. (B) Those receptors that bind TGF-β family proteins or members of the activin family phosphorylate Smads 2 and 3. These Smads can complex with Smad 4 to form active transcription factors. A simplified version of the pathway is shown at the left. Modified from (Zhu et al., 2001). (C) The structure of the large latent TGF-β complex. Modified from (Saharinen et al., 1996) (*297*).

TGF-β is released by platelets, macrophages and neutrophils which are present in the initial phases of the repair process (*298-300*). The growth stimulatory action of TGF-β appears to be mediated via an indirect mechanism involving autocrine growth factors such as PDGF-A and –B, basic FGF or CTGF (*287, 301-303*). TGF-β is a potent stimulatory signal for fibrosis, and elevated TGF-β mRNA or protein levels have been documented in several different fibrotic diseases and during wound repair (*287, 304*). The stimulation of fibroplasia *in vivo* has been attributed to several documented *in vitro* activities of TGF-β including stimulation of fibroblast proliferation, stimulation of the synthesis of extracellular matrix components including fibronectin, type I collagen and integrins, and stimulation of the synthesis of protease inhibitors and gelatinases and suppression of stromelysin gene expression (*305, 306*). In epithelial cells, TGF-β arrests the cell cycle in late G1 (*307*). The growth inhibitory response of epithelial and endothelial cells to TGF-β lies on the transcriptional control of key regulators of the cell cycle by the incoming TGF-β signal (*308*) (*309*).

TGF-β1 is the most abundant isoform in all tissues and in wound fluid most of the TGF-β is the type 1 isoform (*6, 310*). However, in vitro studies have suggested that the different TGF-β isoforms may play both distinct and non-redundant functions during wound healing (*311*). All three genes of TGF-β isoforms share a similar intron/exon structure with a total of seven exons (Table II) (*93*). The TGF-β1 promoter does not contain TATA or CAAT elements, includes several response elements important in wounding (*312*) (Fig. 8.). Expression of TGF-β1 is induced in response to various mediators and by autoinduction which is mediated through AP-1 sites in the TGF-β1 promoter (*312*).

TGF-β is secreted from cells in a latent, inactive complex containing two proteins: active TGF-β and its prodomain, TGF-β latency-associated protein (LAP). Active TGF-β is cleaved from its propeptide, but it remains associated with TGF-β by non-covalent interactions, conferring latency to the complex (*313*). Two chains of pro-TGF-β associate to form a disulfide bonded dimer. LAP and TGF-β together form the small latent TGF-β complex. Most cell lines, however, secrete also large latent TGF-β complexes, containing additional high molecular weight proteins that associate with LAP. Best characterized of these are latent TGF-β binding proteins (LTBP's), which can bind to LAP via a disulfide bond(s) (Fig. 9. C). LTBP appears to increase the efficiency of secretion of TGF-β from cells and promotes the association of TGF-β to matrix and facilitates its activation (*305*). Several ECM proteins have been suggested to bind the active form of TGF-β1. These include type IV collagen, fibronectin, thrombospondin,

and the core proteins of the ECM proteoglycans decorin and biglycan (*314, 315*). Thrombospondin binds both the active and latent forms of TGF-β, and induces the activation of TGF-β by an unknown mechanism, whereas decorin functions as a negative regulator of active TGF-β (*314, 315*). Cell-surface-mediated activation of the latent complex has been suggested where the activation of the latent complex may be a multifactorial process depending, in part on the mannose-6-phosphate receptor, plasmin and a transglutaminase (*316, 317*). The interaction of TGF-β with α_2-macroglobulin may account for the latency of serum TGF-beta (*318*).

Biological effects of TGF-β are mediated via heteromeric complexes of type I and type II transmembrane serine/threonine kinase receptors (Fig. 9. A and B). Binding of TGF-β to the type I receptors requires coexpression with type II receptor, whereas type II receptors bind its ligand independently. The ligand independent homodimerization of type II receptor makes it possible that it is constitutively autophosphorylated (*309*). The type III receptors include a transmembrane proteoglycan betaglycan and membrane glycoprotein endoglin (*319, 320*). A function has been suggested for the type III receptors as a reservoir for readily available TGF-β that can be presented with high affinity to the type II receptors (*309*).

The Smad family of signal transducer proteins has been identified as mediators of the TGF-β signal from the cytoplasm to the nucleus (Fig. 9. B). Smad proteins can be classified in three groups; receptor-regulated Smads (Smad 2 and Smad 3), common partner Smad (Smad 4) and inhibitory Smads (Smad 6 and Smad 7) (*321, 322*). Activation of TGF-β-dependent gene expression is commonly mediated through Smads 2,3 and 4. Smads 2 and 3 are normally present in the cytosol. Once activated by TGF-β, Smads 2 and 3 interact transiently with type I TGF-β receptor kinase and become phosphorylated. Smad2 and Smad3 then form a heterodimeric complex with Smad 4. These complexes are subsequently translocated to the nucleus and activate expression of target genes, in concert with other nuclear factors the identity of which can vary depending on the promoter and cell type (*323, 324*). Smad7, which prevents the interaction of Smad 3 with the TGF-beta receptor is capable of mediating both autoinhibitory feedback and down-regulation of TGF-β signaling (*325*). Inhibition of TGF-β receptor signaling function by inhibitory Smad 7 may represent an effective and general mechanism to alter the balance between signals with opposing effects on complex cellular responses including inflammation and cell proliferation/cell death (*104*) (*326*). At present, only about twelve genes are known to contain Smad-responsive regions, binding Smad complexes

directly or indirectly (*327*). These genes include proα2(I) collagen and proα2(V) collagen and integrin beta5 (*106, 328*). In Smad 3 null fibroblasts, no TGF-β-driven transactivation of the promoters of COL1A1, COL1A2, COL3A1, COL5A2, COL6A1 and COL6A3 was observed (*327*). In addition, TGF-β-mediated induction of CTGF requires Smad 3 in fibroblasts (*261*). Mice null for Smad 3 show accelerated cutaneous wound healing characterized by an increased rate of re-epithelialization and a reduced local inflammatory infiltrate (*329*).

In the repair process, TGF-β1 is one of the first cytokines to elicit inflammatory cell recruitment (*11*). In addition to platelets, TGF-β is secreted by all of the major cell types participating in the repair process. TGF-β1 can be chemotactic and mitogenic for neutrophils, lymphocytes, monocytes, macrophages, and fibroblasts (*6*). TGF-β1 and lymphocytes can enhance the initiation of inflammatory activity of macrophages through monocyte recruitment (*330*) and macrophage activation (*331*). Inflammatory cells synthesize and secrete additional TGF-β1, which at higher concentrations may induce the expression of its own gene and other growth factors, thereby increasing the cellularity of the wound. The role of TGF-β1 during the proliferative phase is the ability to stimulate angiogenesis and collagen deposition in tissues (*291, 332*). In the maturation phase of healing, TGF-β1 may continue to exert control over extracellular matrix components, also by inhibiting the actions of those substances that would otherwise serve to break them down. TGF-β1 plays a role in some apoptotic processes, which occur during wound maturation (*333*) (*39*).

PDGF

Tissue culture work had shown that a factor released from platelets upon clotting was capable of promoting the growth of various types of cells (*334, 335*). This factor was subsequently purified from platelets and given the name platelet-derived growth factor (PDGF). PDGF is produced by a number of cell types besides the platelets such as fibroblasts, keratinocytes, skeletal myoblasts, vascular smooth muscle cells, endothelial cells and macrophages. PDGF is a major mitogen for fibroblasts, smooth muscle cells, and other cells and act mainly on connective tissue cells (*256*).

PDGF is a positively charged hydrophilic protein which exists in three forms. Each form consists of a homo- or heterodimeric combination of two genetically distinct, but structurally related, polypeptide chains designated A and B. The subunits are linked by disulfide bonds (*256*). Each chains of human PDGF is synthesized as a propeptide from which N-terminal pro-sequence is removed after synthesis. The mature B-chain can remain at the cell surface by the

hydrophobic stretch of the C-terminal part of chain but C-terminal proteolytic processing may also occur for the B chain (*336*). Splice variants exist for the A chain (*95, 97, 98*). In human platelets, PDGF AB and PDGF AA isoforms are the most common isoforms (*337, 338*). Among other cell types, there are examples of cells making only the A or B chain and of cells making both PDGF chains (*256*). All possible isoforms, PDGF-AA , PDGF-BB and PDGF-AB are biologically active (*5*). Two novel members of the PDGF family were recently identified, i.e., PDGF-C (*339*) and PDGF-D (*340*).

Chromosomal localization and gene structure for both chains have been determined (Table II). The PDGF-A and B genes have seven exons. The B chain gene is identical to the human c-sis gene (*101, 341*). The activity of both genes is regulated independently of each other in some cell types and co-ordinately in some other cell types (Fig. 8.) (*256*). The synthesis of PDGF can be induced by IL-1, IL-6, TNF-alpha, TGF-beta and EGF (*256*).

The PDGF isoforms exert their cellular effects by binding to the specific transmembrane receptors. Two distinct human PDGF receptor proteins have been identified, PDGF α-receptor (*342, 343*) and PDGF β-receptor (*344, 345*). The two receptor proteins are structurally related and consist of an extracellular portion containing five immunoglobulin-like domains, a single transmembrane region, and an intracellular portion with a protein-tyrosine kinase domain. A functional PDGF receptor is formed when the two chains of a dimeric PDGF molecule (homo- and heterodimer) each bind one of the above receptor molecules, resulting in their approximation, dimerization and activation (*346*). Binding of the ligand leads to the formation of receptor/ligand aggregates that are internalized by the cell. PDGF αR binds each of the three forms of PDGF dimers with high affinity (*342*). Although PDGF βR binds PDGF-BB with high affinity, it has not been reported to bind to PDGF-AA (*347*).

Receptor binding by PDGF activates intracellular tyrosine kinases, leading to autophosphorylation of the cytoplasmic domain of the receptor as well as phosphorylation of other intracellular substrates (*348*). Two receptor molecules of the receptor dimer phosphorylate each other. An array of signal transduction molecules interact with α- and β-receptor (*348-350*). Some of them seem to bind with different affinities to the α- and β-receptors, suggesting that the particular response of a cell depends on the type of receptor it expresses and the type of PDGF dimer to which it is exposed (*349, 350*). The synthesis of PDGF receptors is a subject of autoregulation by PDGF (*256*).

The various isoforms of PDGF have different mitogenic and chemotactic activity. Vascular smooth muscle cells (SMC) and fibroblasts express both α- and β- receptors. On SMC, PDGF-AA initiates cellular hypertrophy, while BB induces hyperplasia (*351*). On fibroblasts, the BB isoform initiates chemotaxis, while AA inhibits chemotaxis (*352, 353*). PDGF binds to several plasma proteins and also to proteins of the extracellular matrix which facilitates local concentration of the factor. The factor functions as a local autocrine and paracrine growth factor (*256*). PDGF may act as an immunomodulator by up-regulating intercellular adhesion molecule 1(ICAM-1) in SMC, and inducing transient IL-2 secretion in T cells, and down-regulating of IL-4 and IFN-γ production (*354, 355*).

PDGF is one of several factors that stimulate the healing of soft tissues (*10, 356*). PDGF is a potent mitogen for connective tissue cells, and in addition, it stimulates chemotaxis of fibroblasts, SMC, neutrophils, and macrophages (*256*). PDGF has the ability to activate macrophages to produce and secrete other growth factors of importance for various aspects of the healing process. PDGF stimulates the production of fibronectin (*357*) and hyaluronic acid (*358*) by fibroblasts. PDGF might be important in the later remodeling phase of wound healing, since it stimulates the production and secretion of collagenase in fibroblasts (*359*).

Fibroblasts and SMC of resting tissues contain low levels of PDGF receptors. However, the PDGF-β receptor is up-regulated in conjunction with inflammation, for example, thereby making cells to response to PDGF (*360-362*). In addition to expression of PDGF-β receptors on connective tissue cells after cutaneous injury, expression has also been noticed on epithelial cells (*363*). PDGF has a weak angiogenic activity and PDGF receptors are missing from endothelial cells of large vessels. Instead, they exist on capillary endothelial cells and on microvascular pericytes (*364, 365*). However, PDGF may stimulate angiogenesis in an indirect way, by inducing the secretion of endothelial cell growth factors by myofibroblasts (*366*). Anyhow, the angiogenic effect of PDGF is weaker than that of other growth factors, e.g., of the FGF family (*367*).

CTGF

Connective tissue growth factor (CTGF) belongs to the CCN (Connective tissue growth factor/Cysteine-rich 61/Nephroblastoma overexpressed) protein family (*368, 369*) (Table VI and Fig. 10.). The prototypic members of this family were discovered in the early 1990s and were initially classified as immediate early gene products or growth factors (*370-372*). These highly conserved cysteine-rich proteins share four conserved modular domains with sequence

similarities to insulin-like growth factor binding protein (IGF-BP), von Willebrand factor, thrombospondin, and a cysteine knot characteristic of some growth factors, including PDGF, nerve growth factor, and TGF-ß (*368*).
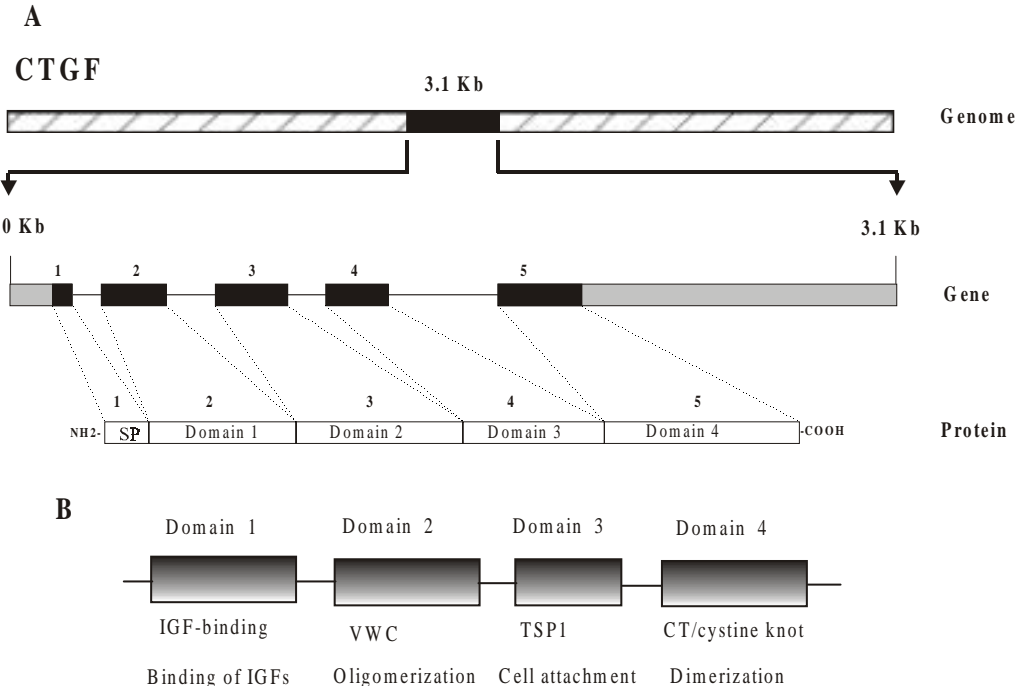

Figure 10. Gene and modular protein structure of CTGF. Modified from (Gupta et al., 2000) (*373*).

Table VI   Summary of structural features of the Connective tissue growth factor/Cysteine-rich 61/Nephroblastoma overexpressed (CCN) family members. Modified from (Gupta et al., 2000)(*373*)

Abbrevi-ation	Full name	Alternative nomenclautre	Modular structure	Protein homologia vs hCTGF	MW protein in human
CTGF	Connective tissue growth factor	Fibroblast-inducible secreted protein-12 (FISP-12, mouse) Transforming growth factor-β-inducible early gene in mouse AKR-2B cells-2 (βIG-M2, mouse) IGF-BP8, IGFBP-rP2, HBGF-0.8, Hcs24 ecogenin	4 Domains	100 %	38 kD
Cyr61	Cysteine-rich 61	Transforming growth factor-β-inducible early gene in mouse AKR-2B cells-1 (βIG-M1) CEF-10 (chicken) IGF-BP10, IGFBP-rP4	4 Domains	43.6 %	40kD
NOV	Nephroblastoma overexpressed	IGF-BP9, IGFBP-rP3 novH	4 Domains	49.0 %	32 kD
WISP-1	Wnt-induced secreted protein 1	Expressed in low-metastatic type 1 cells (ELM-1)	4 Domains	39.0 %	
WISP-2	Wnt-induced secreted protein 2	rCOP-1 CTGF-L (Connective tissue growth factor-like) Heparin-inducible CTGF-like protein (HICP) CTGF-3	3 Domains (lackeing CT domain)	29.8 %	~26 kD
WISP-3	Wnt-induced secreted protein 3		4 Domains (lacking 4 cysteines in domain 2)	35.8 % (WISP1/WISP3 42%) (WISP1/WISP2 37%) (WIPS2/WISP3 32%)	39 kD

CTGF was first detected in the medium from cultured endothelial cells and cloned from human umbilical vein endothelial cells (*371*). It was identified by its cross-reactivity with an antibody to PDGF, but was clearly shown to be a separate molecule (*371*). CTGF is involved in diverse autocrine or paracrine actions in many different cell types (*374*). Leukocytes and lymphocytes do not express the CTGF gene (*262, 371*). It has mitogenic activity and mediates cell adhesion, angiogenesis, increased cell migration and induction of apoptosis (*371, 375-378*). CTGF is overexpressed in fibrotic skin diseases such as scleroderma and keloids (*379, 380*) and in human atherosclerotic plaques (*381*). Furthermore, CTGF is highly expressed in the stroma of certain mammary tumors (*382*). In addition to being a potent fibroblast mitogen and chemoattractant, CTGF stimulates fibroblast procollagen and fibronectin protein production. It also influences α5 integrin mRNA levels *in vitro* (*289*). The matrix-stimulating activity of

CTGF and TGF-β distinguishes them from the other growth factors, such as, EGF, FGF and PDGF which do not induce major levels of extracellular matrix proteins (*129, 289, 291, 292*).

CTGF is a cysteine-rich heparin binding peptide. Its chromosomal localization and gene structure have been determined (Table II). The human CTGF gene comprises five exons and four introns (Fig. 10). Each of the exons 2-5 encodes for one domain, which gives the protein typical CCN-protein family modular structure.

CTGF is not expressed in normal dermal fibroblasts unless cells are treated with TGF-β (*262, 371, 383-385*). The induction by TGF-β is cell-type specific, as it occurs in connective tissue cells but not in epithelial cells or lymphocytes (*262, 371, 383*). CTGF expression by cultured fibroblasts is exclusively induced by TGF-β, whereas other fibrotic mediators such as PDGF, EGF, βTGF, and IGF-1 have no effect on it (*287, 385*). The regulation of CTGF expression by TGF-β appears to be controlled primarily at the level of transcription (*384, 385*). The CTGF gene promoter has a TGF-β response element (TβRE) that regulates its expression in fibroblasts but not in epithelial cells or lymphocytes (Fig. 8.)(*385*). This sequence does not resemble the TGF-β response elements described in other genes, including the Smad recognition sequence (*385*). Originally, the up-regulation of CTGF by TGF-β was thought to be solely dependent on this element (*385*). However, the sequences immediately upstream of the TβRE are necessary for TGF-β and TNF-α to modulate CTGF expression (*384*). A putative consensus Smad site on the CTGF promoter has been indentified. Transfection of Smads 3 and 4 into fibroblasts enhance CTGF promoter activity whereas Smad7 suppress TGF-β-induced CTGF expression (*261*).

Also other factors capable of modulating CTGF expression have been reported. Thrombin, coagulation factor FVIIa and factor Xa can induce the expression of CTGF in human fibroblasts (*386-388*). These *in vitro* findings suggest that coagulation proteases promote the production of CTGF by cells at sites of early wound healing. Elevated cAMP prevents CTGF expression in cells treated with TGF-β and could be involved in the termination of its expression (*389*). It has been suggested that, in addition to inducing a fibrotic response, TGF-β induces an "autoregulatory mechanism" by inducing the synthesis of PGE_2, which acts to limit the fibrotic action of TGF-β, perhaps by elevating cAMP (*390*). Moreover, PGE_2 has been shown to inhibit transcription of the CTGF gene (*391*). The mode of action of CTGF is mediated by specific integrins, which have been identified as its cellular receptors (*376, 392, 393*).

CTGF is potent inducer of angiogenesis (*376, 394*). Although the mechanism by which CTGF induces angiogenesis *in vivo* is not known, it has been speculated that growth factors such as TGF-β and FGF-2 might induce angiogenesis, at least in part, through the induction of CTGF in fibroblasts. This could explain the paradox that TGF-β induces angiogenesis *in vivo* but has anti-angiogenic effects on endothelial cells *in vitro* (*395*). A possible explanation is that TGF-β induces CTGF in fibroblasts, which then act upon endothelial cells to induce angiogenesis (*396*). There is *in vitro* evidence that VEGF induces CTGF gene expression in both endothelial cell and pericytes (*397*). Through its effects on CTGF expression, VEGF may have physiological role by maintaining the capillary strength (*397*). In addition, *in vitro* studies have shown that CTGF promotes the adhesion, proliferation and migration of vascular endothelial cells and can induce the tube formation of vascular endothelial cells (*375*). CTGF mediates endothelial cell adhesion and migration through an integrin $\alpha_v\beta_3$ which plays important roles in angiogenesis (*21*). Interestingly, CTGF is a ligand of this integrin (*376*). Whereas CTGF promotes cell survival in microvascular endothelial cells (*376*) it can also cause apoptotic cell death in smooth muscle cells and certain breast cancer cell lines (*378, 398, 399*). High static pressure up-regulates the expression of CTGF in cultured human mesangial cells and high levels of CTGF in turn induced apoptosis in these cells (*400*).

CTGF can support the adhesion of fibroblasts, endothelial cells, epithelial cells, blood platelets, and other cell types (*392*). CTGF itself is mitogenic for fibroblasts (*262*). The kinetics of CTGF expression are unique. A brief exposure of fibroblasts to TGF-β is sufficient to induce a prolonged high level of CTGF expression (*385*). CTGF does not share all of the biological activities of TGF-β. CTGF does not inhibit epithelial cell growth and modulate immune response (*289*). In addition to its role as a downstream mediator of many of TGF-β's actions on target cells, CTGF can also act synergistically with TGF-β (*262*).

OUTLINE OF THE PRESENT STUDIES

The formation of new and regenerating tissue requires the coordinate regulation of various genes. These encode both structural and regulatory molecules which participate in cell growth and tissue organization. A tight balance between connective tissue synthesis and breakdown is required for the normal functioning of tissues. In normal wound healing, a network of negative feedback mechanisms activated after successful healing is responsible for the proper termination of the proliferative and fibrotic processes, thus restoring tissue integrity. The aim of this study was to analyze the connective tissue remodeling in normal wound healing in purpose to better understand mechanisms which underlie fibrotic processes. The tissue that grows in subcutaneously implanted viscose cellulose sponges were used as wound healing model in rat for the studies.

From many different collagen types, type I and III collagens are the most important and best studied structural components in wound healing, but little is known about the minor interstitial type V collagen. Therefore the present study aimed first to reveal the role of the type V collagen in relation to type I and III collagens in the formation of new connective tissue in experimental granulation tissue. Matrix metalloproteinases comprise an important group of matrix-degrading enzymes. Type V collagen, but not type I or III collagen, is substrate to matrix metalloproteinase -2 and –9 and in addition these proteolytic enzymes degrade gelatins, fragments of other types of collagens. Therefore these gelatinases were chosen for the study the spatial and temporal expression and activity of matrix degrading enzymes. Growth factors regulate both the synthesis and the degradation of matrix molecules. From these regulating factors transforming growth factor β (TGF-β) has the broadest range of activities in tissue repair process. Its downstream mediator in fibroblasts is connective tissue growth factor (CTGF). The spatial and temporal expression of these growth factors together with platelet-derived growth factor (PDGF) –A and –B were studied in the formation of new connective tissue.

The main interest was in the gene expression level of these different molecules. During the course of the present study rat specific hybridization probes for type V collagen, MMP-2 and MMP-9 were prepared.

MATERIALS AND METHODS

Animals

Male inbred PWG rats (250-300 g in weight and 3-5 months of age) were used for the experiments. All rats were kept under standard conditions and were fed rat chow and water *ad libidum*. The animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH). The study was approved by the Committee for Experimental Research of the Helsinki University Central Hospital and the regional authorities.

Experimental wound healing model

Wound repair processes follow a specific time sequence and can be temporally categorized into three major groups: inflammation, tissue proliferation, and tissue remodeling (Fig. 1.). A standardized experimental wound model which follows these sequences was used (*47*). Experimental granulation tissue was induced by implanting the viscose cellulose sponges (5x5x10 mm, dry weight ~ 10 mg; Cellomeda Oy, Turku, Finland) into subcutaneous pockets in the backs of the animals, anaesthetized with midazolam (Dormicum®) and fentanyl-fluanisone (Hypnorm®), as described previously (*48, 51*). The animals were sacrificed on days 3, 5, 7/8, 14, 21, 30, 59 and 84 after implantation. Three sponges from each rat were used as parallel specimens for each analysis. Sponges for the analysis of hydroxyproline and DNA were frozen in liquid nitrogen and stored at -70 °C until used. Sponges for RNA isolation were placed in extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.5% Na-lauryl sarcosine, 0.1 M 2-mercaptoethanol) and stored at -20°C until used. Sponges for immunohistochemical sections were embedded in Tissue Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlandes), frozen immediately in liquid nitrogen, and saved at -70°C until used (Paper I-III). Specimens for histological and immunohistochemical (Paper IV) staining and *in situ* hybridization were placed in 10% normal buffered formalin solution.

Histological staining for collagens

Formalin-fixed tissue was embedded in paraffin and 8-μm sections were stained with hematoxylin-eosin, Weigert van Gieson and toluidine-blue.

Hemoglobin

Hemoglobin was determined as cyanmethemoglobin as recommended by the International Committee for Standardization in Haematology, whereby all hemoglobin forms in the blood

were determined: reduced hemoglobin, oxidized hemoglobin and methemoglobin (*401*). The samples were homogenized in distilled water and were centrifuged at 4°C and 35 000 g for 1 hour. One ml of cyanide solution was mixed with 2 ml of supernatant, and the intensity of the color was measured after 10 min. at a wavelength of 540 nm against the reagent blank. The millimolar extinction 11.5 of cyanmethaemoglobin was used in the calculation of the results.

Determination of total tissue collagen and DNA
The granulation tissues were homogenized in distilled water and the hydroxyproline content was determined from the homogenate as described earlier (*402*). The homogenates were hydrolyzed in 6 N HCl at 130°C for 3 h, the HCl was evaporated and the hydroxyproline content was measured as pyrole, which reacts with p-dimethylbenzaldehyde. The value obtained for hydroxyproline was then used to estimate the total collagen per milligram of wet tissue, assuming that hydroxyproline comprises 13.7% of collagen by weight (*403*). DNA was extracted from the homogenate using the Schmidt-Thannhauser procedure (*404*). Nucleic acids were first treated with alkali at 37°C 20 h, DNA was separated from RNA by centrifugation and hydrolyzed in perchloric acid at 90°C for 30 min. to obtain deoxiriboses, which were measured with diphenylamine using the method of Burton (*405*).

Collagen extraction, identification, and quantitation
Sponges (about 200 mg wet wt) were finely minced and placed in 20 ml of 0.5 M acetic acid containing the protease inhibitors N-ethylmaleimide (8 mM), phenylmethylsulfonyl fluoride (1 mM), and ethylenediaminetetraacetic acid (20 mM). Collagen was dissolved by sequential treatment with 0.5 M acetic acid for 18 h at 4°C and pepsin (100 µm/ml) at 4°C for 6 h. The insoluble material was removed by filtration and the filtrate was neutralised with Tris buffer (50 mM, pH 7.0) and NaOH to inactivate the pepsin. The supernatant was dialyzed three times against 0.5 M acetic acid for 24 h and the collagen was precipitated by addition of NaCl to 4.5 M and centrifugation at 30 000 g. The collagen was then redissolved in 0.5 M acetic acid and again dialyzed against 0.5 M acetic acid to remove salt. Collagens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a urea buffer and a 5% acrylamide separating gel with a 3% stacking gel (*406*). Protein bands were visualized by staining with Coomassie Blue and the relative abundance of collagen α-chains was determined by scanning bands using a Hewlett Packard model ScanJet 4c/T scanner and densitometric analysis was by Intelligent Quantifier™ (Bio Image Systems Corporation, MI, USA). The

amount of type I, III and V chains was estimated by measuring the density of those bands in unreduced sample. The relative abundance of type V collagen α-chains in the collagen preparation extracted from each tissue sample was calculated as a percentage of total collagen α-chains in the sample: type V (%) = V/(I+III+V) x 100.

Extraction of metalloproteinases and gelatinolytic activity assay
The tissue samples were weighed and homogenized in 30 volumes of 50 mM Tris-HCl/0.15 M NaCl/100 mM $CaCl_2$, pH 7.5 and then centrifuged at 10000 xg at 4°C for 15 min. The supernatant was dialysed against 50 mM Tris-HCl buffer/0.15 M NaCl, pH 7.5, and used as enzyme source in zymography. Zymography was performed in SDS-polyacrylamide slab gel, containing 10% acrylamide and 0.1% gelatin as substrate (*407*). After electrophoresis, SDS was removed from the gels with 50 mM Tris-HCl/5 mM $CaCl_2$ for 2x 10 min at room temperature. The gels were then incubated in 50 mM Tris-HCl/5 mM $CaCl_2$/1 µM $ZnCl_2$/1% Triton X-100 at 37°C for 18 h to activate metalloproteinases. Proteins were stained with Coomassie blue G250. High Molecular Weight Standards and Low Molecular Weight Standards (Pharmacia LKB, Biotehnology, Uppsala, Sweden) were used for electrophoresis. Supernatant from the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated HT 1080 human fibrosarcoma cell line was used as positive control for gelatinolytic activity (kindly provided by Dr Karl Tryggvason, Karolinska Institutet, Stockholm). Enzymatic activity was determined by scanning bands using a densitometer (HP ScanJet IIc Scanner, Hewlett-Packard Co, Edina, MN, USA) connected to a computer to measure the area produced by each peak (software, BioImage, Ann Arbor, MI, UK).

Primary antibodies
Rabbit polyclonal antibodies raised against human type I collagen (anti-ICTP) (*408*) and human type III collagen (anti-PIIINP) (*409*) were kindly provided by Dr. J. Risteli (Oulu) and human type V collagen (*410*) was kindly provided by Dr. M. Lehto (Tampere). The antigen for this antibody was prepared from neonatal rat skin, and the antibody was purified by passing it repeatedly through the type I and III collagens immunoabsorbent columns to remove cross reacting antibodies. Anti-human α-smooth muscle (α-SM) actin, clone 1A4 was obtained form DAKO A/S (Glostrup, Denmark). Anti-human MMP-9 was kindly provided by Dr Timo Sorsa (Department of Periodontology, University of Helsinki, Helsinki) (*411*). Pan-specific TGF-β antibody, anti-human CTGF and anti-human PDGF BB were obtained from RD Systems

(Minneapolis, MN, USA). Anti-PDGF A was obtained from Genzyme Diagnostics (Camridge, MA, USA).

Immunohistochemistry

Cryostat sections were stained with an avidin-biotin-peroxidase-complex technique (*412*), using a commercial Vectastain Elite ABC Kit from Vector Laboratories (Burlingame, CA, U.S.A.). Five-micron thick sections were cut from the frozen sponges, fixed in acetone for ten minutes at room temperature and air dried. The sections were consecutively incubated with the blocking serum for 15 min. followed by overnight incubation with the primary antibodies, biotinylated secondary antibody for 30 minutes and peroxidase-labelled ABC for 30 min. All dilutions were made in phosphate-buffered saline (PBS), pH 7.2, and all incubations were performed in humidified chambers at room temperature. Between each step in the staining procedure the slides were rinsed in PBS. The color reaction was developed by an incubation of 15 min. in a 3-amino-ethyl-carbazole solution (0.2 mg/ml in 0.05M acetate buffer containing 0.03% perhydrol, pH 5.0). Finally, the sections were counterstained in Mayer´s haematoxylin and mounted in aqueous mounting medium (Aquamount, BDH, Poole, UK). Negative controls remained in blocking serum instead of the primary antibody. Paraffin sections were stained with the same method as the cryostat sections by little modifications: sections were pre-treated in micro-wave oven, and Tris-buffered saline (TBS), pH 7.4, instead of PBS buffer, was used. Staining was judged for intensity on a scale of 1-5, in addition, in paper II, the area of the loose and dense connective tissue considered to be positive was estimated as a percentage of the total area of the sponge and these two values were taken together. The percentage of positive fibroblast-like cells for α-smooth muscle (SM) actin from all fibroblasts were calculated in paper I. In paper IV, the percentage of positive fibroblast-like, positive rounded (macrophage-like) and positive blood vessel cells of total cells of the sponge were estimated and staining was judged for intensity on a scale of 1-5, these two values were multiplied with each other to form the staining index.

In Situ **hybridization**

5-μm paraffin sections of formalin fixed granulation tissue were placed on Superfrost (Menzel-Gläser, Braunschweig, Germany) slides. The slides were deparaffinized and hydrated through descending ethanol concentrations. Pretreatment included incubation with 10 μg/ml proteinase K at 37°C for 30 min and 4% paraformaldehyde post-fixation. Slides were dehydrated through

ascending ethanol concentrations. For hybridization each labeled RNA-probe was diluted with hybridization solution (20 mM Tris-HCl buffer, pH 8.0, 5 mM EDTA, 0.3 M NaCl, 50% formamide, 10% dextran sulfate, 1xDenhardts's solution, 200 µg/ml sheared herring sperm DNA, yeast tRNA 200 µg/ml, and DEPC-treated water) to the concentration of 1ng/µl, 30 µl per slide. Slides were heated to 95°C for 10 minutes, and cooled on ice. Hybridization was performed in a humidified chamber at +50°C for 18 hours. Slides were washed: once in 5x standard saline citrate (5xSSC at 50°C, for 60 min), once in 2xSSC/50% formamide (at +65°C for 30 min), three times in NTE-buffer (0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA at 37°C for 10 min), treated with RNase A (20 µg/ml in NTE buffer) at 37°C for 30 min, and washed once in NTE-buffer at 37°C for 15 min, once in 2xSSC/50% formamide at 65°C for 30 min, once in 2xSSC and then in 0.1xSSC at room temperature for 15 min each. Digoxigenin-labeled probes were detected following the methods from DIG-detection Kit (Boehringer Mannheim, Mannheim, Germany) using an anti-digoxigenin antibody of dilution 1:2000 and incubation at room temperature for 30 min. Color substrate incubation was for 16 h. Slides were counterstained with hematoxylin and coverslipped with aqueous-based mounting medium. Staining was accepted as positive when it was seen with the antisense but not in the sense probe. Staining was judged for intensity on a scale of 1-5, and in addition, the area of the tissue considered to be positive was estimated as a percentage of the total area of the sponge. The percentage of positive cells of total cells of the sponge were estimated and, in addition, staining was judged for intensity on a scale of 1-5. These two values were multiplied with each other to form the staining index.

Analysis of mRNA

Total RNA was extracted from granulation tissue using the method of Chomczynski and Sacchi (*413*). Aliquots of total RNA (10 µg) were fractionated by electrophoresis on agarose gels and transferred onto MagnaGraph Nylon Transfer Membrane (Micron Separations Inc., USA) by blotting (PosiBlot, Stratagene, La Jolla, CA, USA). Prior to transfer, the integrity of the RNA and the equal loading of the gels were verified by visualising ribosomal RNA subunits with ethidium bromide staining. The filters were UV-crosslinked (Spectrolinker, Spectronics Corporation, Westbury, NY, USA), prehybridized and hybridized with the cDNA probes labelled by random priming (Random Primed DNA Labelling Kit, Boehringer Mannheim, Mannheim, Germany) using [^{32}P]dCTP. The hybridization was performed in 50% formamide, 1 M sodium chloride (NaCl), 1% sodium dodecyl sulphate (SDS), 5xDenhardt's

solution, 10% dextran sulphate and 100 µg/ml herring sperm DNA at 42°C for 16-18 h. The filters were washed three times with 2xSSC (1xSSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at room temperature, and twice with 0.2xSSC and 0.1% SDS at 55°C, and the bound probe was detected using autoradiography at -70°C using Kodak X-omat X-ray films.

Slot blot hybridization was employed for accurate quantitation of different mRNAs. Serial dilutions of the total RNAs were dotted onto nylon filters (MagnaGraph Nylon Transfer Membrane) using a vacuum manifold (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). Dilution series of purified DNA inserts of cDNA probes, denatured by boiling for 10 min, were applied onto the filters as standards. The filters were hybridized and washed as described above. The amounts of specific mRNAs were estimated by scanning the exposed films using a densitometer (HP ScanJet IIc Scanner, Hewlett-Packard Co., Edina, MN, USA), connected to a computer to quantify the bands (software, Bio Image, Ann Arbor, MI, USA). The results were corrected for minor variations in the amount of GAPDH expression in the samples. The copy numbers were calculated using standard curves obtained with serial dilutions of the insert DNAs. To allow comparison of the relative expression of genes coding for the genetically distinct collagen types, the densitometric units per g total RNA dotted were corrected for sizes of the cDNAs (*414*).

Probes

Probes used in Northern blot, slot blot and *in situ* hybridizations are listed in Table VII. For in situ hybridizations, plasmids containing inserts of probes were linearized with restriction enzymes to create templates for unidirectional synthesis of digoxigenin-labeled RNA probes with specific promoters, following the methods from the Riboprobe Synthesis Kit (Boehringer Mannheim, Mannheim, Germany). Transcripts were checked on a 1 % agarose gel, and concentrations were determined by serial dilution color reaction against known concentrations of control-labeled RNA. Hybridization with a Northern blot of granulation tissue RNA confirmed the probe specificity.

Table VII Probes used in this work

Probe	cDNA clone	Probe covers bases	Probe size bp	Specificity	Reference
Type I collagen	pα1R2	3725-4324	600	rat Col1a1	(*415*)
Type III collagen	pRGR5	1706-2217	512	rat Col3a1	(*416*)
Type V collagen	pRCVA1	4751-5310	560	rat Col5a1	(*417*)
MMP-2	pRMP2-2	1552-2451	900	rat MMP-2	(*418*)
MMP-9	pRMP9	529-1048	520	rat MMP-9	(*419*)
MMP14		884-1582	699	human MMP-14	(*420*)
TIMP-2			663	human TIMP-2	(*421*)
CTGF			1000	human CTGF	(*371*)
PDGF-A		774-1078	305	rat PDGF-A	(*422*)
PDGF-B		999-1612	614	human PDGF-B	(*423*)
TGF-β1		1267-1564	298	rat TGF-β1	(*424*)
GAPDH	pRGAPDH			rat GAPDH	(*425*)

Preparation of rat probes for type V collagen and MMP-2 and -9

Total RNA was extracted from rat 7- and 14-day granulation tissue using the method of Chomczynski & Sacchi (*413*). mRNA was prepared from total RNA using the mRNA Purification Kit (Pharmacia LKB, Biotehnology, Uppsala, Sweden) and employed as a template to synthesize cDNA using the First-Strand cDNA Synthesis Kit (Pharmacia LKB, Biotehnology, Uppsala, Sweden) and oligo dT as primer. This first strand was subsequently used as a template in PCR amplification of 40 cycles of denaturation (95°C for 1 min.), annealing (55°C for 1 min.), and extension (72°C for 2 min.). cDNA fragments corresponding to nucleotides 4969-5528 in the carboxy propeptide region of human type V collagen (*426*) were amplified using forward primer (5´-ATCCAGGACGCGGCGGAACATCG-3´) and reverse primer (5´-GGAAGCGGAGGGCCTTGTCGTAGC-3´). For rat MMP-2, cDNA fragment (clone pRMP2-2) corresponding to the C-terminal part of mouse MMP-2, nucleotides 992-1883 (*427*), was amplified using forward primer (5´-ATGAGAGCTGCACCAGCGCCG-3´) and reverse primer (5´-GATTTGATGCTTCCAAACTTCACGC-3´). For rat MMP-9, the cDNA fragment (clone pRMP-9) corresponding to the central part of mouse MMP-9, nucleotides 526-1043 (*232*), was amplified using forward primer (5´-GGAGACGGTATCCCTTCGACGG-3´) and reverse primer (5´-ACGCACAGCTCTCCTGCCGA-GTTGC-3´). The PCR products were cloned in Bluescript vector (Stratagene, La Jolla, CA, USA). The PCR products were subjected to electrophoresis on a 1% agarose gel (1xTAE), purified using Geneclean Kit (BIO 101 Inc., La Jolla, Ca, USA) and ligated into the *Eco*RV-cut and T-tailed (*428*) pBluescript KS+ vector (Stratagene, La Jolla, Ca, USA). Cloned PCR products were sequenced using an automatic

sequencer, Model 373A (Applied Biosystems, Inc., Foster City, CA, USA). Comparison of the nucleotide sequence of the rat clone of proα1(V) collagen with the corresponding human and hamster sequences revealed 88.7 % and 94.8 % homology respectively (*426*). At the level of deduced polypeptide, identities of 96.5 % and 98.2 % were observed between rat and human and rat and hamster respectively. Comparison of the nucleotide sequence with other rat collagen types was possible for type I collagen, type II collagen and type III collagen (*416*), homology being 64 % (203/317), 65% (194/300) and 61% (128/209), respectively. Clone pRCVA1 detected two mRNAs of approx. 6.1 and 6.9 kb in size in Northern hybridization.

Statistical analysis
One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple-comparison test was used for comparisons between the different times of observation. The data were analysed using NCSS 2000 software (SxST, Arezzo, Italy). P values of less than 0.05 were considered statistically significant. The data are presented as mean ±sd.

RESULTS

Sponge implants [I and II]

The mean wet weight of the implants increased up until day 7, after which it declined slowly. The mean wet weight of all the samples was 196 mg (the range 100 - 347 mg). The ingrowth of granulation tissue, determined as percent ingrowth area of the total sponge area on cross-section image, increased gradually, reaching 100% on day 30. In the early phase of the granulation tissue development there was mostly loose, cellular connective tissue at the periphery of the cellulose sponge and the central parts of the sponge were more or less acellular. With time the connective tissue became more mature with a fibrillar configuration. Simultaneously the granulation tissue spread to the central portions of the cellulose sponge.

The phenotypes of the fibroblast-like cells in developing granulation tissue were analysed by immunostaining of α-SM actin. The walls of the arterioles, venules and capillaries were positive at every time point, whereas elongated fibroblast-like cell staining varied with time. The staining intensity of α-SM actin increased up to day 14, when 34 % of fibroblasts-like cells were positive for α-SM actin (Fig. 11. A). Thereafter, the staining declined clearly and was negligible after day 30.

Hemoglobin analyses were carried out to get information on the extent of vascularity in the granulation tissue (Table VIII). A sharp increase in the hemoglobin content occurred from day 8 onwards with peak values seen on days 8 and 14 (1 mg/sponge mean wet weight). The increase in the hemoglobin concentration correlated with the growth of capillaries as seen at
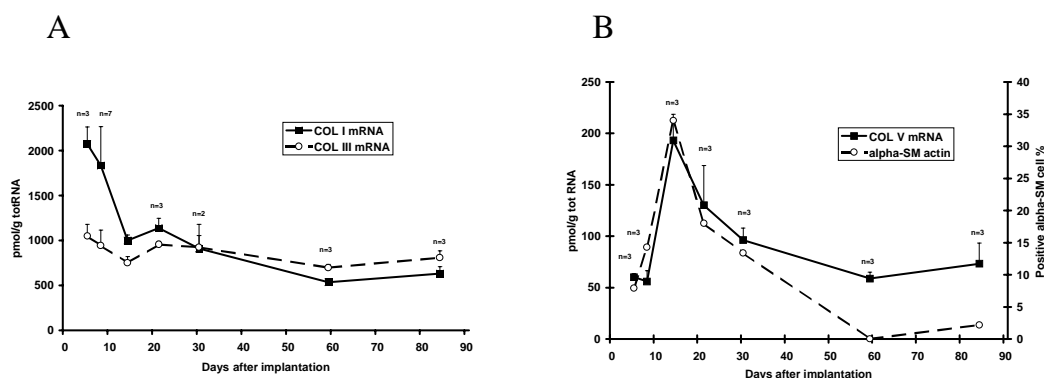
A

B

Figure 11. Changes in expression of, proα1(I) (A), proα1(III) (A) and proα1(V) (B) mRNAs as a function of time in developing granulation tissue. The slot blots were subjected to densitometric scanning and the values are given as pmol per g total RNA. Each points represents the mean ±sd of n sponges (n values are indicated in figure). The percentage of cells positive for α-SM actin from total fibroblasts as seen in immunohistochemistry is shown together with type V collagen mRNA (B).

histology. The hemoglobin content of the sponges declined slowly up to day 30, after which time it remained at a level which was about 30% of the peak value. The DNA content in the sponges indicates the number of cells in the granulation tissue (Table VIII). DNA content increased evenly from day 5 onwards reaching its peak value on day 21, after which time DNA content remained at the same level.

Table VIII   Changes in total collagen, DNA, hemoglobin and type V collagen content in rat granulation tissue over period of 84 days after implantation.

Days after implantation	Total collagen mg/sponge			Total DNA µg/sponge			Total Hb mg/sponge			Purified type V col % extractable collagen		
	mean	±sd	n	mean	±sd	n	mean	±sd	n	mean	±sd	n
3	0.2	±0.1	3	176	± 89.2	3	0.3	±0.1	3	6.8		1
5	0.7	±0.4	3	140	± 14.7	3	0.4	±0.3	3	4.0	±2.6	2
8	2.3	±1.0	8	265	± 90.3	8	1.0	±0.4	9	9.4	±1.7	5
14	3.5	±0.9	6	302	± 44.2	6	1.0	±0.5	5	7.7	±1.8	3
21	3.6	±1.2	6	414	±105.4	6	0.9	±0.2	5	10.5	±4.7	6
30	4.5	±1.4	6	437	± 45.8	6	0.6	±0.2	5	10.7	±3.8	6
59	5.9	±4.0	3	423	±180.6	3	0.4	±0.1	2	nd		
84	5.3	±0.2	3	426	± 51.8	3	0.3		1	8.8		1

Values are the mean ±sd of n specimens as indicated in table.
nd = not done

Interstitial collagens in granulation tissue [I and II]

The collagen contents determined as hyroxyproline, rose most rapidly between days 5 and 8 (range: 0.7 to 2.3 mg/sponge mean wet weight) and continued to increase up to day 59 (5.9 mg/sponge mean wet weight) (Table VIII). Separation of purified interstitial collagens by SDS-PAGE demonstrated that the major collagen types were I, III and V (Fig. 12., Table VIII). SDS PAGE was conducted under non-reducing conditions. Type V collagen was observed on the gels predominantly as two α-chains - α1 and α2, with only trace amounts of α3. The proportion of type V collagen from the two other collagens increased with the time, reaching a level of about 10% of soluble collagen by day 8 and remaining at that level up to day 84.

In loose and mature connective tissue, type I and III collagens were expressed at protein level throughout the experimental period. Staining for type III collagen was most intense between days 8 and 21, whereas staining for type I collagen was most intense from the day 30 onwards. Type V collagen protein was expressed weakly from day 30 onwards in the loose connective tissue. In the blood vessel walls, type V collagen was expressed strongly and the total level of type V collagen protein staining increased in parallel with the increasing amount of blood vessels, whereas very little expression of type I and III collagens was detected.

Since there was no rat specific probe for type V collagen available, a cDNA clone (pRCVA1) for rat proα1(V) collagen mRNA was constructed. This clone detected two mRNAs of approx. 6.1 and 6.9 kb in size in Northern hybridization and no cross-hybridization to proa1(I) and proa1(III) collagen mRNAs was observed. The quantitative changes in the expression of proα1(I), proα1(III) and proα1(V) mRNAs as a function of time were studied by slot blot hybridization (Fig. 11. A and B). The three collagen types were expressed at every time point. Proα1(V) mRNA expression was much lower than that of the other two fibrillar collagens (about 10% of the expression of the type I collagen mRNA). The expression maximum was seen on day 14 and thereafter it declined to the level of day 5. Proα1(III) mRNA expression was quite stable throughout experiment. Proα1(I) mRNA expression was highest on days 5-8, and thereafter its expression remained at the same level as type III collagen expression.

To evaluate which cells synthesize proα1(I), proα1(III) and proα(V) collagen mRNAs, *in situ* hybridization was used (Table IX). Cells producing type I, III and V collagens were found at every time point measured. Differences in the expression of collagen types were seen in the number of positive cells, localization of positive cells relative to extracellular matrix type (loose or mature) and in cell types. The expression for type I collagen was the most prominent and type V collagen the weakest. Expression of type I collagen was seen mostly in fibroblast-like (spindle-shaped) cells, but some positive rounded, macrophage-like cells were also seen,
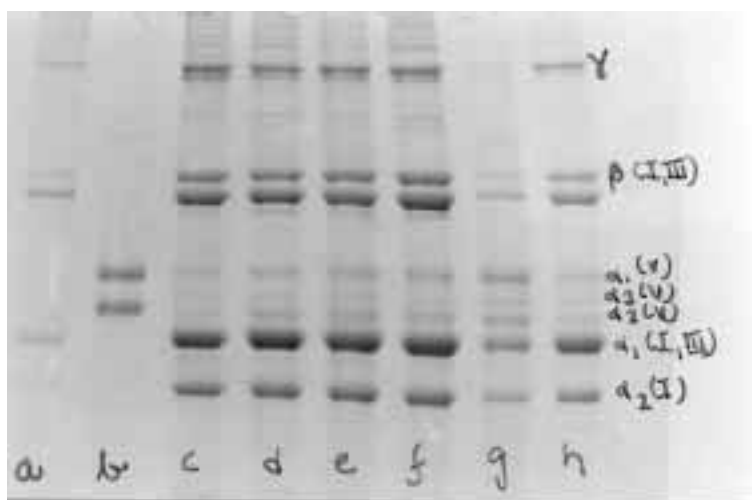

Figure 12. SDS-PAGE profile showing collagen types in rat granulation tissue. Identification of the collagen bands was confirmed by their comigration with appropriate standards; lane a: type I collagen, lane b: type V collagen (human placenta,), lane c - h: soluble collagens from granulation tissue, day 7 (lane c), day 14 (lane d), day 21 (lane e), day 30 (lane f), day 84 (lane g) and day 84 (lane h). Collagens were extracted in acetic acid, containing pepsin and electrophoresis was performed under nonreducing conditions.

especially at the later stages. The expression of type III collagen followed a pattern similar to that of type I collagen. However expression of type III in the macrophage-like cells was more prominent than that of type I collagen. Type V collagen was seen in the macrophage-like cells especially on days 21-84. Few positive cells formed cell clusters which resemble the nucleus of new capillaries. In addition, type V collagen expression was seen on early days also in some endothelial cells and pericytes in a fibroblast-like configuration. Most positive cells for all three collagen types were situated in the loose connective tissue, especially type V collagen mRNA, but type I and III collagen mRNA positive cells were found also in the mature connective tissue in considerable amounts on days 14-30.

Table IX Changes in procollagen mRNA expression in the fibroblast-like and macrophage-like cells of the developing granulation tissue.

Days after implantation	proα1(I) collagen mRNA		proα1(III) collagen mRNA		proα1(V) collagen mRNA	
	Fibroblast-like cells	Macrophage-like cells	Fibroblast-like cells	Macrophage-like cells	Fibroblast-like cells	Macrophage-like cells
3	0.2	0.5	0.1	0.2		
5	4.0	2.0	4.0	2.0	3.0	0.8
8	10.8	1.2	7.2	4.8	2.4	0.8
14	7.8	1.2	5.4	2.7	1.0	0.8
21	10.6	1.1	5.3	1.5	0.0	2.0
30	24.0	0.0	6.6	0.6	4.5	4.5
59	14.0	3.0	12.0	6.0	0.6	5.4
84	3.0	6.0	6.0	6.0	1.2	2.8

The area of the tissue considered to be positive was estimated as a percentage of the total area of the sponge, and this was considered together with the intensity of staining in the fibroblast-like and macrophage-like cells.

MMP-2 and MMP-9 in granulation tissue [III]

Four detectable gelatinolytic activity bands were detected by zymography. The most prominent band was the latent form of MMP-2. The amount of latent MMP-2 did not change during the experimental period (Fig. 13. A). The active form of MMP-2 increased up to day 14, after which it remained constant. MMP-9 at a MW 92 kD was observed only on days 3-7 and was considered to be the latent form of MMP-9 (Fig. 13. B). A MW 76 kD was not present in the control and was not identified. It did not show any apparent changes during the experimental period.

Strong expression of MMP-9 protein was detected by immunohistochemisty in the polymorphonuclear leukocytes and macrophage-like cells on days 3-8, and thereafter only a low levels of MMP-9 expression could be seen in the more mature granulation tissue. Only occasional some fibroblast-like cells positive for MMP-9 were detected. For quantitation of MMP-2 and MMP-9 mRNAs, slot blot hybridization was used (Fig. 14. A). MMP-2 mRNA expression was quite stable until day 30, whereafter it started to increase, becoming significantly higher ($p<0.05$) on day 59 than on day 8, and on day 84 the expression was significantly higher ($p<0.05$) than on days 5-30. The expression of the MMP-9 gene was minimal on days 14 and 21 and started to increase after 4 weeks of granulation tissue formation. On day 59 it was significantly higher ($p<0.05$) than on days 8-30.

To evaluate which cells synthesize MMP-2 and MMP-9 mRNA, *in situ* hybridization was used. Expression of MMP-2 mRNA was seen at every time point, mostly in fibroblast-like cells. The expression of MMP-2 mRNA appeared to increase somewhat in the later stages of development of granulation tissue. The expression of MMP-9 mRNA was low in the early stages of formation of granulation tissue and detectable in both macrophage-like and fibroblast-like cells. At later time points, the expression was exclusively in macrophage-like cells. To study the activation of MMP-2, the MMP-14 and TIMP-2 mRNA expression during normal wound repair by Northern blot analysis was examined (Fig. 14. B). The MMP-14 gene was expressed at every time point and showed maximal expression on day 8. However, no statistically significant difference was found between the different time groups,
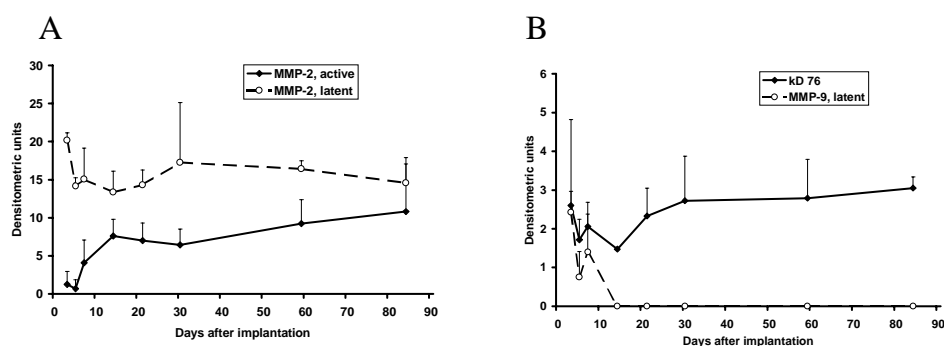

Figure 13. Gelatinolytic activity of rat granulation tissue measured as densitometric units from zymography. In A results of MMP-2 active and latent form are given and in B results of MMP-9 latent and 76 kD form. Each points represents triplicate results from different days after implantation.

owing to the high standard deviation. TIMP-2, which is secreted as a complex with proMMP-2, showed fairly constant mRNA levels up to day 30 and thereafter increased in the more mature granulation tissue, the expression of TIMP-2 being significantly ($p < 0.05$) higher on day 84 than on days 5-30.
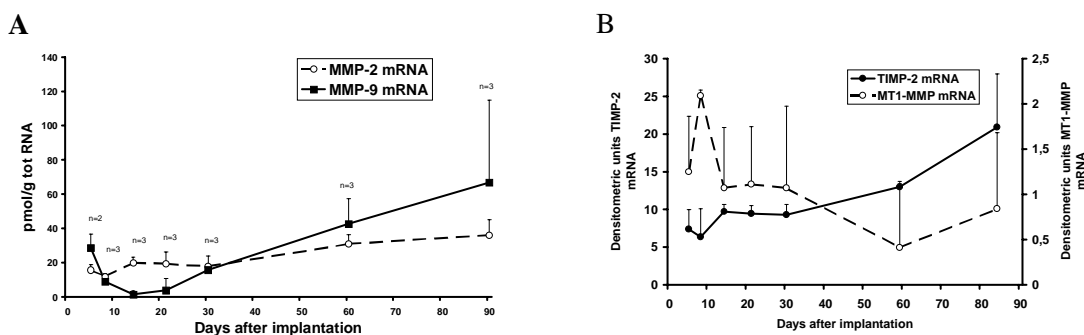

Figure 14. Changes in expression of MMP-2 and MMP-9 mRNAs (A) as a function of time in developing granulation tissue. The slot blots were subjected to densitometric scanning. The values are given as pmol per g total RNA. Changes in expression of TIMP-2 mRNA and MMP-14 mRNA (B) as a function of time in developing granulation tissue. The Northern blots were subjected to densitometric scanning. The values are given as densitometric units. Each point represents the mean ±SD of 2-6 sponges.

Growth factors in granulation tissue [IV]

To evaluate which cells synthesize CTGF mRNA, *in situ* hybrization was used. On days 5 and 7, the most intense staining of CTGF was seen at the periphery of the granulation tissue. CTGF positive cells were mainly pericytes and some endothelial cells of blood vessels. In the newly formed granulation tissue within the cellular sponge, cells expressing CTGF mRNA were clearly seen during days 5-14, mostly in the fibroblast-like cells (Fig. 15. A). There were also some positive endothelial cells, especially on day 7. On day 21, when the granulation tissue within the sponge was not yet totally filled by cells, the expression of CTGF was restricted to cells near the sponge empty space. Thereafter, there was only occasional expression of CTGF mRNA, both in fibroblast-like and in macrophage-like cells. Among the fibroblast-like cells positive for CTGF, there were some cells with dendritic extensions of the cytoplasma.

To determine the sites of expression of TGF-β1, CTGF, PDGF A and B at the protein level in developing granulation tissue, immunohistochemistry was used. The staining patterns for TGF-β, PDGF AA and BB protein were quite similar (Fig. 16. A,B and C). On days 5 and 7, cells staining most intensely were fibroblast-like cells, with expression decreasing towards the end

of the process of granulation tissue development. In addition, TGF-β, PDGF AA and PDGF BB were found in rounded cells (macrophage-like) throughout the experiment period. Some blood vessel cells had also positive staining for these growth factors at the periphery of the granulation tissue especially on days 5 and 7. CTGF protein expression differed from that of the other growth factors in that the amount of positive cells was much lower (Fig. 15. B). In addition, intense staining was observed only on days 5 and 7, and, although all three cell types were positive, blood vessels were stained most strongly. Another antibody specific for mouse CTGF was used to confirm the immunohistochemical staining. Staining pattern was similar with both antibodies except that the mouse specific CTGF did not immunostain macrophage-like cells.

To quantify the mRNA expression of growth factors, Northern hybridization were used (Table X). PDGF A and B mRNAs were expressed in the rat granulation tissue at every time point measured, without any significant differences. The maximum expression for PDGF A was on day 14 and for PDGF B on day 21. The expression of both TGF-β1 and CTGF mRNA was seen at every time point during granulation tissue formation, although with slightly different expression patterns. Maximum expression of CTGF mRNA expression occurred during the first two weeks, with the expression on days 7 and 14 significantly ($p<0.05$) higher than that on day 60. There were no significant time-related differences in the expression of TGF-β1, but the strongest expression was seen during the first two weeks.

Table X Changes in expression of CTGF, TGF-β1, PDGF-A and PDGF-B mRNAs as a function of time in developing granulation tissue.

Days after implantation	CTGF			TGF-β1			PDGF-A			PDGF-B		
	mean	±sem	n	mean	±sem	n	mean	±sem	n	mean	±sem	n
5	1.5	±0.2	3	7.0	±0.8	3	1.4	±0.3	3	1.6	±0.2	3
7	2.6	±0.2	3	8.3	±0.2	3	2.1	±0.2	3	3.1	±0.4	3
14	2.9	±0.5	5	8.4	±0.6	5	2.7	±0.5	6	2.4	±0.3	2
21	1.1	±1.1	3	4.8	±3.3	3	1.7	±0.7	4	4.3	±0.7	3
30	1.2	±0.3	6	6.0	±1.5	6	1.3	±0.1	6	3.1	±0.8	6
60	0.4	±0.3	6	4.0	±1.4	6	2.4	±0.7	5	3.2	±0.6	6

Northern blots were subjected to densitometric scanning. The values are given as densitometric units.
Values are the mean ±sem of n specimens.
n = number of specimens.

A
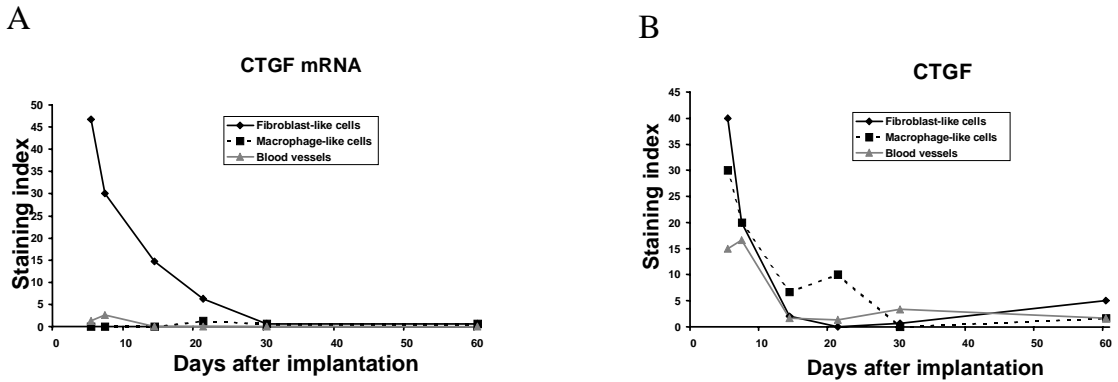
CTGF mRNA

B

CTGF

Figure 15. Expression of CTGF mRNA analysed with the *in situ* hybridization method (A) and expression of CTGF protein analysed by immuno-histochemistry (B) in developing granulation tissue. Staining index is explained in materials and methods.
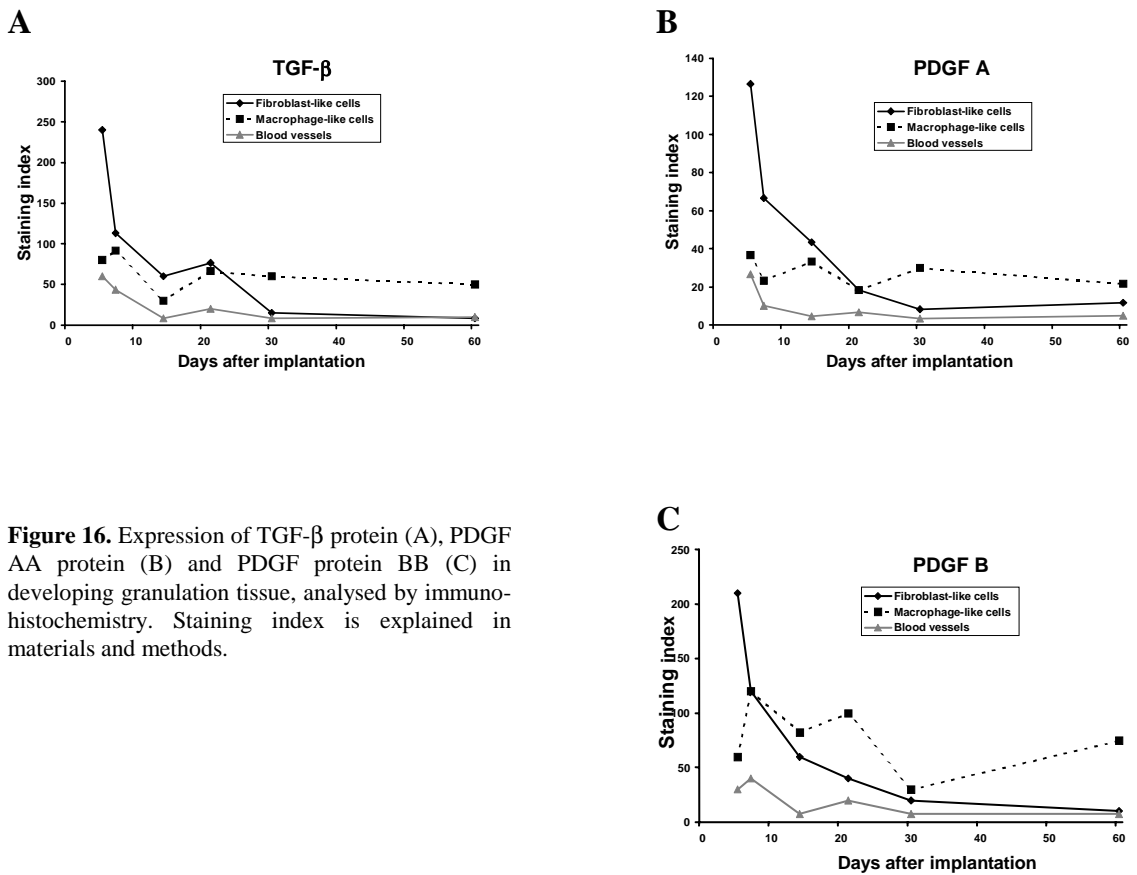
A

TGF-β

B

PDGF A

Figure 16. Expression of TGF-β protein (A), PDGF AA protein (B) and PDGF protein BB (C) in developing granulation tissue, analysed by immuno-histochemistry. Staining index is explained in materials and methods.

C

PDGF B

DISCUSSION

Wound healing is a highly controlled cascade of events were different cells, ECM components and biologically active hormonal effectors act to regain tissue integrity. The process is continuous, but is in general divided into three partly overlapping phases: inflammatory, proliferative and remodelling phase. If the loss of tissue is large, healing proceeds via formation of granulation tissue and contraction (*429*). During the formation of granulation tissue, macrophages, fibroblasts and new blood vessels grow into the wound space in a coordinated manner. Their interdependence is illustrated by the release of cytokines by macrophages that stimulate fibroblasts to synthesize an extracellualar matrix. This extracellular matrix serves to support cell and vascular ingrowth carrying nutrients to sustain the cellular functions (*430*). In physiological wound healing, a network of negative feedback mechanisms activated after successful healing is responsible for the proper termination of the proliferative and fibrotic processes, thus restoring tissue integrity (*431*).

In the current work attention has been paid on the developing of the granulation tissue; to investigate the interstital collagen deposition, especially type V collagen and their degradation and factors which stimulate these functions. Experimental granulation tissue induced by subcutaneous viscose cellulose sponge implants offers a means for studying the development of granulation tissue. The granulation tissue that develops in a viscose cellulose sponge is similar both chemically and histologically to the tissue formed in a healing wound (*47*).

In the present study, the basic parameters such as total collagen, DNA and hemoglobin, in the developing granulation tissue were first investigated. The results were in general agreement with previous studies using rat granulation tissue (*47, 432*). The amount of hemoglobin in the granulation tissue appears to reflect the magnitude of the capillary bed (*47, 433*). In the current study, however, hemoglobin values decreased after the proliferative phase, but the capillary bed did not diminish and the blood vessels were present throughout the duration of the experiment (II). The reason for this discrepancy could be a reduction in blood flow. In developing granulation tissue a decrease in regional blood flow after proliferative phase has been demonstrated (*434*). Granulation tissue fibroblasts (myofibroblasts) develop microfilament bundles and the expression of α-SM actin. TGF-β1 induces α-SM actin expression in granulation tissue myofibroblasts and it is transiently expressed by myofibroblasts during experimental wound healing (*30, 210*). We describe here that α-SM actin was temporarily expressed in fibroblast-like cells, the most prominent expression being in

the proliferative phase of wound repair, when cell number and collagen deposition increased. With these background results, we studied the temporal and spatial expression of the different collagen types and factors that are important in the regulation of ECM remodeling, such as MMP-2,-9,-14 and TIMP-2 and growth factors CTGF, TGF-β1 and PDGF.

Type I, III and V collagens in wound healing
Following tissue injury, ECM restoration has to be achieved by a controlled *de novo* synthesis as well as degradation of damaged ECM molecules. Although the ECM contains a large number of glycoproteins, those belonging to the family of collagen probably play the most important role, since they not only provide the structural scaffold of the tissue but also regulate many cellular functions. In soft tissue repair most important collagen types are type I and III collagen. The function of type V collagen is not clear, but it may be a part from the provisional extaracellular matrix during wound healing and acts in cell attachment processes and angiogenesis. Type V collagen is synthesized by endothelial and smooth muscle cells in vitro (*158, 193, 435*). Increased amounts of type V collagen on injured endothelium of aorta and neointima of the aortic prosthesis has been found (*198, 436*). The presence of type V collagen in blood vessel walls implies its potential role in angiogenesis and hemostasis. Many studies have shown that type V collagen prevents platelet aggregation and adhesion in vitro (*195, 437*). It has been postulated that the function of type V collagen on the endothelial cell surface could be that of a passive barrier to platelet aggregation and that, once these barriers are breached and interstitial collagens and/or other thrombogenic molecules are exposed, platelet aggregation will occur (*193*). Type V collagen has been shown to be an important matrix component in different wound healing models (*438, 439*). The high vascularity of wound healing may partially account for increased amounts of type V collagen. The increase of type V collagen coincides with the increase of the new blood vessels in granulation tissue and the ratio of type V to type I collagen is higher in the initial stage of wound healing (*438, 439*).
We describe here that type I, III and V collagen mRNAs and proteins were expressed over a period of three months in developing granulation tissue (I and II). Type V collagen mRNA had a clear peak expression in the proliferative phase of wound repair, which correlated with α-SM actin expression. These results suggest that some myofibroblasts may be responsible for type V collagen expression. The percentage of type V collagen mRNA of the three collagen types correlates well with the protein levels of the corresponding collagen type, suggesting that regulation occurs at the transcriptional level, which differs from the suggested regulation of

type I and III collagen. Other results described on experimental granulation tissue model indicate that translational control mechanisms are important in type I and III collagen mRNA regulation (*151, 416*). Type I and III collagen mRNA levels were high throughout the experiment. Since type III collagen levels in the blood vessels are relatively high, increased vascularity of the granulation tissue could contribute to the high levels of type III collagen (I and II).

Although the level of type V collagen protein was low compaered to the two other collagen types, it constituted a very significant proportion of the total collagen in the granulation tissue compared with other tissues such as skin and gingiva (*440, 441*). On the other hand, the level of type V collagen protein is in agreement with the level of type V collagen detected in bovine and human aorta (*197, 442*). The protein levels of type V collagen in regard to the time is in agreement with the results others have demonstrated in wound repair model. However, their study revealed lower levels of type V collagen and only α1-chain, whereas in the present study all three α-chains were found (*432*). This discrepancy might be due to the different granulation model (stainless steel wire mesh cylinders vs. cellulose) and different extraction process.

The most prominent cell types in the granulation tissue synthesizing type I, III and V collagen mRNAs were fibroblast-like and macrophage-like cells. Cultured fibroblasts have been shown to synthesize type I and III collagens but not type V collagen (*193*). The active synthesis of each collagen types was seen during the whole period of repair process. Some endothelial cells in early granulation tissue were positive for type V collagen. In the current study and earlier reports have demonstrated that type V collagen at the protein level is strongly associated with blood vessel wall during repair processes, pinpointing to its importance in angiogenesis (*193, 198*). However in mRNA level this was not so clear, especially in the later phase of the formation of granulation tissue, where no mRNA expression was seen in endothelial cells. Elongated fibroblast-like cells and macrophage-like cells were the most prominent types of cells expressing type V collagen mRNA in the later granulation tissue. Positive fibroblast-like cells were seen mostly in the loose connective tissue.

Type I and III collagen proteins in the developing granulation tissue were seen exclusively in the interstitium of the granulation tissue, demonstrating their important role in providing structural scaffold of the tissue. In contrast, type V collagen protein was expressed intensively in the blood vessel walls, and only in the later phase of granulation tissue some expression was seen also in the loose connective tissue. However, in control material from rat intact small bowel, type V collagen protein in the blood vessels was almost non-existing, suggesting that

type V collagen expression is associated with newly formed capillaries and that it may have a function in angiogenesis. Type V collagen epitopes are very often masked *in situ* and therefore difficult to detect immunologically, due to their incorporation with collagen I fibrils, at least in mature tissues (*80, 175*). This suggests that type V collagen might not be readily available to cells in all situations. However, during tissue remodeling or in neoplasia, where the new synthesis of collagen occurs, accumulation of type V collagen has been observed, and it is possible that it could become transiently available to cells and fulfill a specialized function, such as to act in cell attachment processes and angiogenesis (*180, 443*).

MMP-2 and MMP-9 in wound healing

Activity of MMPs is a requirement for the remodelling of the extracellular matrix during wound healing. Experimental granulation tissue provides an opportunity to study the kinetics of the appearance of these enzymes. In collagen degradation, these enzymes can act after collagenases (MMP-1 and MMP-8) have degraded collagen types to gelatins, which are the main substrates for MMP-2 and MMP-9. Their genes are expressed during dermal wound healing (*286*). In contrast to collagenases these enzymes have also the ability to degrade native type V collagen. Because of our interest in the developing granulation tissue, we focused this work on enzymes expressed in the dermis. MMP-1, which is also important in wound healing, is expressed mostly by epithelial cells, whereas MMP-8 is expressed mostly by neutrophils and in the early phase of wound healing (*444, 445*).

In the present study we found that whilst both gelatinases of the MMP family, MMP-2 and MMP-9, were active, their genes were expressed quite differently during granulation tissue development. The levels of the latent form of MMP-2 were unaltered during the granulation formation, whereas the amount of the active form of MMP-2 increased as the granulation tissue developed. Fibroblasts produce MMP-2, which is consistent with the finding that the active form of MMP-2 starts to increase at the time when fibroblasts start to invade the healing wound (*285, 286*). In agreement with the notion that MMP-2 expression is constitutively expressed, MMP-2 mRNA expression was seen throughout the formation of granulation tissue (III). The cells responsible for this expression were mostly fibroblast-like cells.

MMP-14 and TIMP-2 are required for the activation of latent MMP-2 (*266, 276, 277*). MMP-14, TIMP-2 and pro-MMP-2 form a ternary complex at the cell surface. TIMP-2 combines with MMP-14 to form a receptor for the latent MMP-2 and that free MMP-14 may then activate the latent MMP-2 by proteolysis. Excess TIMP-2 interferes with this activation

mechanism by binding and inhibiting all available MMP-14 molecules (*252*). In the present study we found that MMP-14 and TIMP-2 mRNA are expressed during normal wound repair (III). MMP-14 gene was upregulated during the first week of wound healing, which coincides with an increase in MMP-2 activation, as shown by gelatin zymography. In contrast, the TIMP-2 gene was constitutively expressed, like the MMP-2 gene. Only in the later phase of the repair process was the TIMP-2 mRNA level slightly higher than at other time points, implying the completion of the wound healing process.

The gelatinolytic activity of MMP-9 was seen only in the early phase of the formation of the granulation tissue and in its latent form. The cells responsible for this activity were polymorphonuclear leukocytes and macrophages, which was shown by immunohistochemistry (III). These cells are probably invaders from the surrounding tissues and already have proenzyme in their granules, because no clear increase in MMP-9 gene expression was seen at these time points. Both cell types store MMP-9 in granules and these cells are important in the early wound healing process, after which they disappear and fibroblast proliferation continues (*268*). MMP-9 gene expression is apparently inducible, because it started to increase towards the end of the experiment but with no detectable concomitant gelatinolytic activity. This suggests that MMP-9 regulation occurs at the post-transcriptional level. Another explanation may be that MMP-9 is retained in the tissue and may still be active in the pericellular environment (*446*).

Macrophage-like cells were the most prominent cell types to express MMP-9 mRNA. Elevated expression of MMP-9 mRNA in the mature granulation tissue might contribute to the downregulation of the wound healing process. In the same wound healing model myofibroblasts started to disappear after one month of the developing granulation tissue (I). These two events might be part of an apoptotic pathway resulting in the cessation of the wound healing process (*235*). Growth factors and mediators of inflammation regulate the expression of the genes, which encode these enzymes in a cell-type specific manner (*224, 255, 447*). In addition, contact with the collagenous extracellular matrix may enhance MMP-2 and MMP-14 expression in dermal fibroblasts by activating a signalling pathway (*205*). The findings of the present study are consistent with the previously reported observation that MMP-9 gelatinolytic activity is detectable only during the early days of wound healing and mostly in its latent form (*286*). Their model was a skin wound, and they demonstrated that MMP-9 activity was from granulation tissue and that the mRNA expression was mostly in the epithelial cells. The wound

healing model presented here lacks the epidermis which could explain why only low expression of MMP-9 mRNA was found during the early days of wound healing (III).

CTGF, TGF-β and PDGF in wound healing

The formation of new and regenerating tissue requires the coordinate regulation of various genes which encode both regulatory and structural molecules that participate in cell growth and tissue organization. This process proceeds in a cascade fashion. Initiating factors need not be continuously present and may function by activating an autocrine or paracrine system to control connective tissue formation. Once the genes encoding the initiators have been activated in a permissive environment, the formation of complex structures proceeds in a programmed fashion no longer dependent on the presence or action of the initiator. Numerous studies have demonstrated that TGF-β is likely to be one of the initiator cytokines for both normal wound repair and fibrotic disorders (*291, 304*). Recently CTGF has been found to act as a mediator of many of the effects of TGF-β on connective tissue (*371*) (*262*). In the current work we demonstrate that the temporal and spatial expression of CTGF mRNA and protein is more restricted than the other fibrogenic growth factors, TGF-β1 and PDGF, in the development of granulation tissue.

The expression of CTGF mRNA and protein were seen during the two first weeks of the repair process (IV). It was associated with strong expression of TGF-β1, the only known factor to induce CTGF expression. CTGF mRNA was predominantly expressed on fibroblast-like cells. In addition, during the first week, the most intense expression was observed in the periphery of the granulation tissue, mainly in pericytes and in some endothelial cells of blood vessels, indicating that blood vessels from the surrounding host tissue were penetrating into the new granulation tissue. Expression of CTGF mRNA and protein was generally found in the same cells.

Previous studies have demonstrated maximal expression of CTGF mRNA early in wound healing and that only connective tissue cells express CTGF mRNA (*287, 289, 448*). No expression of CTGF has been observed in normal blood vessels *in vivo* or in quiescent endothelial cells *in vitro*, but the factor is markedly upregulated in migrating and proliferating endothelial cells *in vitro* (*379, 449, 450*). CTGF induces neovascularization indicating that it functions as an aniogenic inducer *in vivo* (*376*). More evidence of CTGF being an angiogenic factor comes from *in vitro* studies, which show that CTGF promotes the adhesion, proliferation and migration of vascular endothelial cells and can induce tube formation of vascular

endothelial cells (*375*). In addition, CTGF mediates endothelial cell adhesion and migration through the integrin $\alpha_v\beta_3$, which plays important roles in angiogenesis. CTGF is a ligand of this integrin (*21, 376*). Although the mechanism by which CTGF induces angiogenesis *in vivo* is not understood at present, growth factors, such as TGF-β and bFGF might induce angiogenesis evidently, at least in part through the induction of CTGF in fibroblasts. This could explain the paradox that TGF-β induces angiogenesis *in vivo* but has antiangiogenic effects on endothelial cells *in vitro* (*395*). There is *in vitro* evidence that CTGF gene expression is induced by VEGF in both endothelial cells and pericytes. Through its effects on CTGF expression, VEGF may maintain capillary strength (*397*). Furthermore, smooth muscle cells and some endothelial cells in atherosclerotic plaque vessels, but not in normal arteries, express high levels of CTGF mRNA and protein (*381*). In the present study we found CTGF mRNA in forming blood vessels *in vivo*, suggesting a role for CTGF in neovascularization during wound repair. In addition, CTGF appeared to be involved in the very early stages of connective tissue formation, as indicated by its presence in the "front" of forming connective tissue. This phenomenon can be linked to the ability of CTGF to mediate cell adhesion and stimulate cell migration (*451*). Others have demonstrated that fibroblast adhesion to CTGF through integrin $\alpha_6\beta_1$ leads to reorganization of actin-cytoskeleton, cell spreading and the formation of filopodia and lamellipodia (*451*).

The importance of TGF-β1 to the developing granulation tissue is supported by its high level of expression at both the mRNA and protein level throughout the experimental period (IV). TGF-β1 protein expression was seen in fibroblast-like cells, macrophage-like cells and blood vessels during the whole repair process, with the most intense staining in fibroblasts at the early phase. In chronic granulomatous tissue others have observed TGF-β protein expression in macrophages before and during fibrosis, and at later stages in fibroblasts associated with the area of active fibrogenesis (*452*). On the other hand, improved healing has been demonstrated in mice in which a specific downstream signaling of TGF-β has been interrupted (*453*). Endogenous TGF-β1 may thus actually function to increase inflammation and retard wound closure. CTGF is not only mitogenic and chemotactic for fibroblasts, but it also stimulates the synthesis of at least two extracellular matrix components, type I collagen and fibronectin (*289*). In the present study we found that total collagen synthesis started to increase during the first week (II). These results support the idea that TGF-β could induce matrix synthesis directly and indirectly via CTGF.

PDGF-A and -B protein was observed in many different cell types, important in wound healing, such as in fibroblast-like cells, macrophage-like cells and blood vessels. Moreover, PDGF-A and -B gene expressions were observed in developing granulation tissue over the whole period of granulation tissue formation without any significant time-related differences. This likely reflects the importance of these growth factors in wound healing; by stimulating chemotaxis of many cells at the beginning of the healing and later activating fibroblasts in the formation of extracellular matrix. Upregulation of PDGF-A protein within fibroblasts and capillaries in healing wound has been demonstrated and expression of PDGF-B protein is induced by injury (*360, 454*). PDGF has probably two major but distinct roles in cutaneous wound repair: an early function to stimulate fibroblast proliferation and a later function to induce the myofibroblast phenotype. In the present wound-healing model myofibroblast proliferation peaked after two weeks of the formation of granulation tissue [I]. The timing of the appearance of PDGF in the present study is in agreement with the suggestion that the appearance of myofibroblasts is a late effect of PDGF (IV) (*455*).

The current results support the importance of CTGF, TGF-β and PDGF in the developing granulation tissue. The extreme complexity of the mechanism of action of PDGF and TGF-β reflects their presence in many different cell types during the entire process of granulation tissue development. In contrast, CTGF expression was seen in a more restricted fashion, which supports the idea that CTGF functions as a downstream mediator of TGF-β early in the repair process. The exression results of CTGF indicate that a more detailed understanding of the functions of CTGF will increase our understanding of tissue formation in both normal development, tissue regeneration, reapir and human fibrotic disorders.

CONCLUSIONS

Type I, III and V collagen were observed to be actively expressed at mRNA and protein level in the present wound healing model. Although the amount of type V collagen was lower than the two other interstitial collagen types, type I and III collagen, it constituted a significant proportion of the total collagen. Type V collagen mRNA expression pattern in regard to the time differed from the two other collagens analysed, suggesting some myofibroblasts involvement in type V collagen expression. Thus the function of type V collagen may be a part of the provisional extaracellular matrix during wound healing and act in cell attachment processes and angiogenesis. The data presented in our study support these concepts. Cells expressing type V collagen mRNA were mostly fibroblast-like and macrophage-like cells and some blood vessel cells in the early phase of granulation tissue formation also expressed this collagen type. However, at the protein level, type V collagen protein was observed almost exclusively in blood vessel wall cells during the whole repair process and only occasional type V collagen protein was located in the loose connective tissue and only in the later phase of granulation tissue. In contrary, type I and III collagen proteins localized exclusively in the dense connective tissue.

The findings of this study suggest that gelatinases are present for an extended period of time during tissue repair and that they have different roles in the wound healing process, despite their similar substrate specificity. MMP-2 mRNA was constitutively expressed and mostly in fibroblast-like cells, whereas macrophage-like cells were the most prominent cell types to express MMP-9 mRNA. Its expression was induced and increased along with the developing granulation tissue. Elevated expression of MMP-9 mRNA in the mature granulation tissue might contribute to the downregulation of the wound healing process. The roles of TIMP-2, MMP-14 in granulation tissue development are unclear at present but their presence during the whole period of repair process support their important role in the activation of MMP-2.

The current results emphasize the importance of TGF-β, CTGF and PDGF in the developing granulation tissue. All the growth factors were strongly expressed early in the development of granulation tissue indicating their important role in the induction of matrix synthesis. The extreme complexity of the mechanism of action of PDGF and TGF-β reflects their presence in many different cell types during the entire process of granulation tissue development. In contrast, CTGF expression was seen in a more restricted fashion, which supports the idea that

CTGF functions as a downstream mediator of TGF-β early in the repair process. We observed accordingly that CTGF is involved in angiogenesis.

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