INTESTINAL HEALING AND TUMOUR INVASION. THE ROLE OF COLLAGENOLYTIC ENZYMES

JOS VAN DER STAPPEN

INTESTINAL HEALING AND TUMOUR INVASION
THE ROLE OF
COLLAGENOLYTIC ENZYMES

INTESTINAL HEALING AND TUMOUR INVASION
THE ROLE OF
COLLAGENOLYTIC ENZYMES

een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
maandag 29 november 1993
des namiddags te 3.30 precies

door

Johannes Wilhelmus Josephus van der Stappen

geboren 23 juni 1958 te Heesch

Promotores: Prof. Dr. H.H.M. de Boer
Prof. Dr. J.J.H.H.M. de Pont

Co-promotor: Dr. Th. Hendriks

VOOR LIEFE KARIN

CONTENTS **PAGE**

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

This thesis describes investigations designed to study the potential role of collagenolytic enzymes involved in the healing of intestinal wounds and the growth and spreading of colorectal tumor cells. Although the healing of wounds and invasion and metastasis of tumor cells do not seem to have a lot in common, a detailed analysis of the cellular and molecular events that take place does reveal remarkable similarities [1,2,3]. The loss of regulation of cellular behaviour during carcinogenesis is basically controlled by the same peptide mediators that play an important role in tissue repair and wound healing [4,5]. These peptides (cytokines) have a critical role in the control of collagen degradation, the recruitment and formation of new fibroblasts, the formation of new collagen and other molecules of the extracellular matrix and the formation of new blood vessels, processes common to both carcinogenesis and tissue repair.

Both wound healing and carcinogenesis also feature the coordinated activity of proteolytic enzymes and their natural inhibitors. The consequences of their combined action on extracellular matrices determines to a large extent the outcome of the physiological or pathological process.

Both issues are briefly introduced below with the emphasis on the possible role that proteolytic enzymes of the metalloproteinase class might play. Also, the biology of the extracellular matrix component collagen is briefly reviewed as an understanding of this protein is vital to the work presented.

WOUND HEALING

The proper healing of wounds, after the inevitable damage of connective tissues, is a complex sequence of biological activities which requires the mobilization of a number of different cell types at appropriate time intervals. For simplification wound healing has been divided into three distinct, but overlapping phases: inflammation, granulation

tissue formation and matrix formation and remodelling (Figure 1).

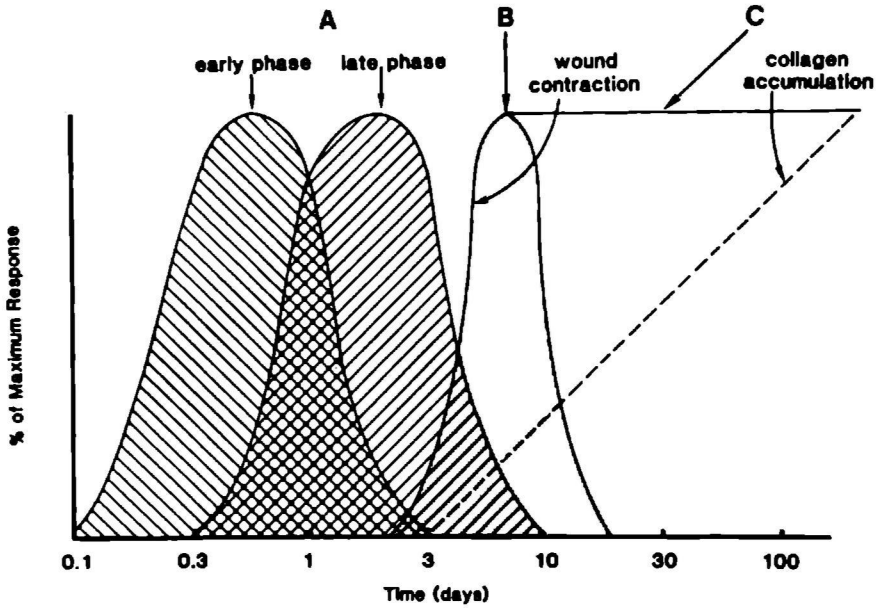

Figure 1: Phases of wound repair. Healing of a wound has been arbitrarily divided into three phases: (A) inflammation (early and late), (B) granulation tissue formation, and (C) matrix formation and remodelling. Those phases overlap considerably with one another and are plotted along the abscissa as a logarithmic function of time. Inflammation is divided into early and late phases denoting neutrophil-rich and mononuclear cell-rich infiltrates, respectively. The magnitude of wound contraction parallels granulation tissue formation, as indicated. Collagen accumulation actually begins shortly after the onset of granulation tissue formation, continuing throughout the phase of matrix formation and remodelling. From: Clark R.A.F. Overview and general considerations of wound repair, Chapter 1 pp 3-34. In: The Molecular and Cellular Biology of Wound Repair. Clark R.A.F. and Henson P.M.(eds). Plenum Publishing Coop. New York, 1988. (with permission).

Following injury, blood vessel disruption leads to extravasation of blood constituents, with concomitant platelet aggregation and blood coagulation. Coagulation factors such as collagen and laminin induce the formation of a gel that seals off the exposed area from the environment. This temporary gel consists of fibrin, fibronectin, platelets and entrapped blood cells. Mediators

released by blood coagulation, complement pathways and cell activation or death at the wound site induce the influx of inflammatory leucocytes. They also increase the permeability of undamaged vessels adjacent to the wound resulting in more widespread leakage of plasma proteins. The influx of neutrophils attempts to clear the wound area of foreign particles, especially bacteria, but will cease after the first few days of tissue injury if no wound infection has occurred. However, if contamination has not been cleared effectively the generation of granulocyte chemoattractants will persist and interfere with the next phases of wound healing.

The influx of monocytes and their conversion to macrophages occurs slightly later and marks the beginning of tissue repair. Macrophages phagocytose and digest pathogenic organisms, scavenge tissue debris, including degenerated neutrophils and release a number of biologically active substances such as vasoactive mediators, growth factors and enzymes. Many of these substances facilitate the recruitment of additional inflammatory cells and assist the macrophage in tissue decontamination and debridement. The inflammatory reaction continues with increasing numbers of macrophages accumulating and fibroblasts moving into the gel. The fibroblasts are responsible for the deposition of loose connective tissue and the onset of angiogenesis. This newly formed tissue has a distinct granular appearance and is therefore referred to as granulation tissue. The origin of wound fibroblasts and the signals that induce their proliferation and migration into a wound space are as yet unknown. Fibroblasts migrating into a wound area initially deposit great quantities of fibronectin. Fibronectins are cell-adhesive and matrix-organizing (glyco)-proteins that aid wound healing by stimulating cell attachment and migration and probably function as a template for collagen deposition.

The final phase of wound repair which can continue for many years is the elimination of fibronectin from the matrix and the accumulation of type I collagen fibres that provide the remaining scar with increasing tensile strength.

The elimination of connective tissue debris during the healing process is a fundamental prerequisite in order to

restore normal function. It has been shown that connective tissue degrading enzymes [6,7,8], secreted by infiltrating granulocytes and macrophages and by local mesenchymal cells involved in the healing process, are the means by which the organism achieves this important task.

INTESTINAL WOUND HEALING

Much of our current knowledge on wound healing has been obtained from studies on skin wounds. Although it seems likely that all wounds heal by common mechanisms, a somewhat different physiological environment may cause tissue-specific alterations in the repair process. It has been shown that significant metabolic differences exist between healing in the gastrointestinal tract and skin [9]. Also, collagen production by fibroblasts from colon and skin appears to be affected differently by various regulatory compounds [10]. Thus, one should exert caution in extrapolating the results from research on skin repair to the healing of other soft tissues [11].
The complexity of intra-abdominal healing and the fact that complications that might occur during the healing of a bowel wound are very difficult to diagnose in early stages of healing frequently leads to a prolonged morbidity or even mortality [12]. A multitude of factors such as old age, local infections, the nutritional condition of the patient, oxygen supply to the anastomoses, suture techniques and the location of the anastomosis have been linked to impaired anastomotic healing [13,14].

A dominating factor in the outcome of large bowel surgery is the occurrence and extent of anastomotic leakage. In order to be able to tackle the problems brought about by anastomotic leakage it is necessary to define the parameters that control the onset and progress of this complication. From previous studies, both clinical and experimental, is has become clear that disturbed intestinal healing is mainly confined to the large bowel [15,16]. Structural and functional differences between the small and large bowel therefore might hold the clue to improve large bowel surgery.

It has been known for some time from a number of

experimental studies including both small and large bowel that the submucosa, a thin layer of connective tissue, is crucial to proper healing [17].

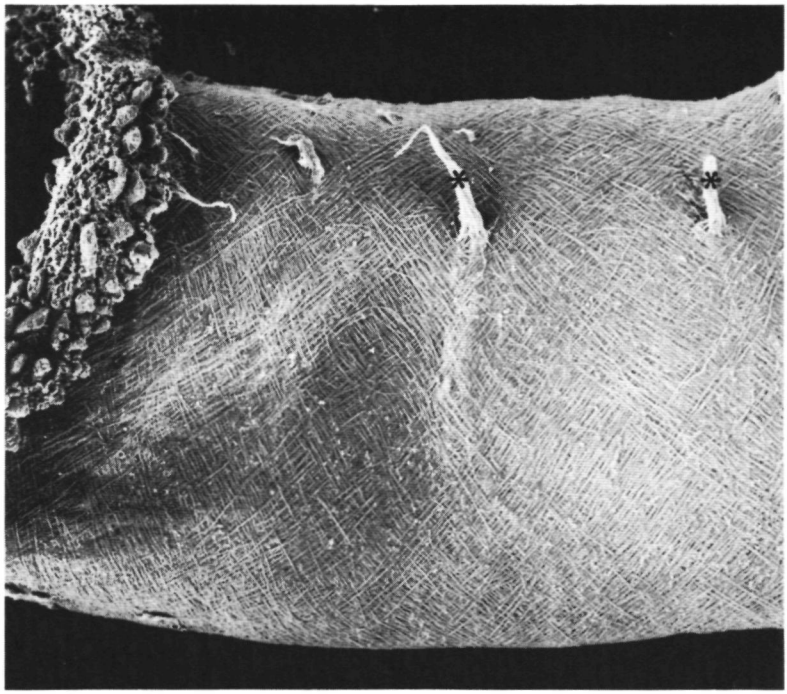

Figure 2: A scanning electron micrograph showing the collagenous framework of the submucosa of the rat small intestine. Collagen fibres are arranged in two sets of helices that interweave. Angles formed by two helices are slightly different, depending on the area of the wall. The everted mucosa with villi is seen in the top left hand corner. From: Komura T. The lattice arrangement of the rat small intestine: scanning electron microscopy. Cell Tissue Res 251: 117-121, 1988.

The submucosa, a connective tissue sheet interspersed between the epithelial layer lining the lumen and the muscular layers surrounding the gut, is composed almost entirely of collagen fibrils (Figure 2, ref.18). It was shown that, in order to construct a proper intestinal anastomosis, sutures have to be anchored in this layer. Based on this observation much of the scientific attention became focused on the collagen metabolism in the anastomosis and its implications for uncompromised healing. Collagen

levels in healing wounds have been quantified by measuring hydroxyproline, an amino acid residue unique to collagens. From these studies the hypothesis was generated that during the first post-operative days the restoration of the so called "collagenous equilibrium" is vital for the bowel to regain its original strength [19].

Investigations in animal models have shown that during the first day after operation the anastomotic collagen concentration is massively and transiently reduced [for review, see 11]. It has been inferred that (localized) collagen degradation occurs in the anastomotic area in the early post-operative period. Indeed, a certain amount of resorption of the extracellular matrix appears to be an integral part of tissue repair in general [20]. Subsequently, *de novo* collagen synthesis overtakes degradation and restores the collagen content to - or above - pre-operative levels. It has been shown recently in our laboratory that anastomotic collagen synthesis may be significantly increased already within one day after operation [21]. A delayed or only partial recovery of pre-operative collagen levels or a prolonged state of collagen degradation is then hypothesized as the possible cause of loss of anastomotic strength, thereby increasing chances for anastomotic leakage. Although it was recognised in an early stage that excessive collagen loss could be the result of a temporary imbalance between one or more collagenolytic enzymes and their natural inhibitors, virtually no data were available on the presence of collagenolytic enzymes in intestinal anastomoses. Therefore, we have investigated the occurrence of such enzyme activity in the early phase of anastomotic healing in the rat intestine.

COLORECTAL CANCER

Colorectal cancer is a major cause of death in the industrialized world. Figures released by the WHO estimate a total of 570000 new cases worldwide in 1992 [22]. Environmental as well as genetic factors have been associated with the development of large bowel cancer. Large bowel cancer is clearly linked to environmental factors: people living in parts of Africa and Asia have a very low

incidence of this cancer whereas in the industrialized countries it is one of the most common types of cancer. The incidence of colorectal cancer in the general population increases rapidly with age. Peak incidence in both man and woman occurs in the 8th decade of life. The direct relationship of age with the incidence of cancer is generally believed to result from the cumulative effects of carcinogens on colonic cells over a long period of time. If, however, colorectal cancer appears in the first decades of life usually predisposing factors such as familial polyposis syndromes or ulcerative colitis are involved [23].

Most colorectal cancers develop from benign adenomas (polyps) in what is referred to as the adenoma to carcinoma sequence [24]. The progression of colorectal cancer is a multistage process, thought to depend on an accumulation of mutations in a number of genes [25,26]. Cytogenetic analysis has shown that most cancers are chromosomally abnormal and that both oncogenes and tumour suppressor genes are involved in carcinogenesis (reviewed in [27]). As a consequence of the genetic alterations the control of cell proliferation and the preservation of normal structural associations (cell-cell and cell-matrix) are disrupted, thereby creating an environment where normal intercellular interactions may be changed.

A biologically and clinically important difference between benign and malignant colorectal tumours is the ability of the latter to attack extracellular matrices by releasing tissue destructive enzymes. This provides the means for local expansion (invasion) and spreading to distant organs (metastasis) [28,29]. In order to move from the primary site to metastatic sites tumour cells have to cross through several connective tissue barriers. Perhaps the most important of these is the basement membrane that underlies the endothelial cells of the vascular system.

Enzymes belonging to all the major enzyme classes, the aspartic-, serine-, metallo-, and cysteine- proteinases are potentially involved in invasion and metastasis due to their ability to degrade one or more extracellular matrix proteins, activate an enzyme capable of doing so, or inactivate proteinase inhibitors. Latest insights actually propose the concerted action of enzymes of different classes

involved in a so called proteolytic cascade. Metalloproteinases such as type IV collagenase and stromelysin have been strongly implicated in the proteolytic aspect of the invasive process [30,31].

Recently, it was shown for the first time that a tumor cell membrane-associated receptor exists for type IV collagenase [32]. Previously, the existence of a urokinase-type plasminogen activator receptor had been identified on the outer membrane surface of tumor cells [33].

Liotta [28] identified the three main steps in invasion and metastasis as an initial process of attachment of tumour cells to the basement membrane (phase 1) followed by proteolysis of the local connective tissue stroma (phase 2) and finally the migration of tumour cells through the proteolyzed stroma (phase 3). Liotta's model has recently been refined by Mareel et al. [34] who have included factors regulating homo- and hetero-typic cell-cell adhesion dynamics.

An important regulatory mechanism in the control of proteolytic activity is the presence of specific proteinase inhibitors. Loss of inhibitor activity by whatever mechanism invokes a situation that resembles the loss of tumour suppressor genes which will eventually result in an increased malignant potential. A detailed examination of tumor-associated proteolytic events may help to reveal the fundamental mechanisms of tumour invasion and metastasis and disclose possible drug targets. The potential role of interstitial collagenase (MMP-1) has been studied in matched tumour and control samples from a large number of patients diagnosed for colorectal cancer. The results of this study are presented in chapter 5.

THE BIOLOGY OF COLLAGEN: A BRIEF REVIEW

One of the unique properties of multicellular organisms is their organization of cells into cooperative clusters which, when combined, form macroscopical functional units called organs. The cells within tissues are supported by and in close contact with an extended network of extracellular

macromolecules: the extracellular matrix (ECM). Extracellular matrices determine body shape and stability, compartmentalization of organs and several cellular activities. The more commonly used term, connective tissue, refers to the ECM plus the cells found in it. The major cell-type found in the ECM is the fibroblast, responsible for the production of the majority of the extracellular macromolecules. In highly specialized connective tissue structures the ECM is secreted by more specific fibroblast-like cells such as chondrocytes which form the deformable cartilage matrix and osteoblasts which produce the rigid bone matrix. Additional specialized ECM structures are found in tendons, cornea, basement membranes, fasciae and teeth.

The wide interest in ECM research today has been preceded by a period of absolute ignorance, due to a general belief that the ECM served mainly as an inert physical stabilizer of tissues. The regulatory role of the ECM and its cellular components influencing cellular development, proliferation and differentiation of cells, cell motility and metabolism in health and disease, is now generally recognized and a topic of research worldwide.

The main classes of extracellular macromolecules that make up the ECM are collagens, proteoglycans, elastin, fibronectin and laminin.

Within the framework of this thesis attention will be limited to the collagens. For detailed information on the other matrix components the reader is referred to some recent reviews [35-40].

The collagens are a family of highly characteristic (glyco)-proteins, present throughout the animal kingdom. They are the most abundant of mammalian proteins, being distributed throughout the body and constituting 25 to 30 per cent of total body protein.

So far, a family of thirteen distinct collagen molecules, composed of at least 25 polypeptide chains, have been recognized and characterized to varying degrees [41-43] (Figure 3). The remarkable feature of this collagen superfamily is its varied repertoire of molecular structures and patterns of supermolecular assembly. The fibrillar collagen types I, II, III and V and the non-fibrillar

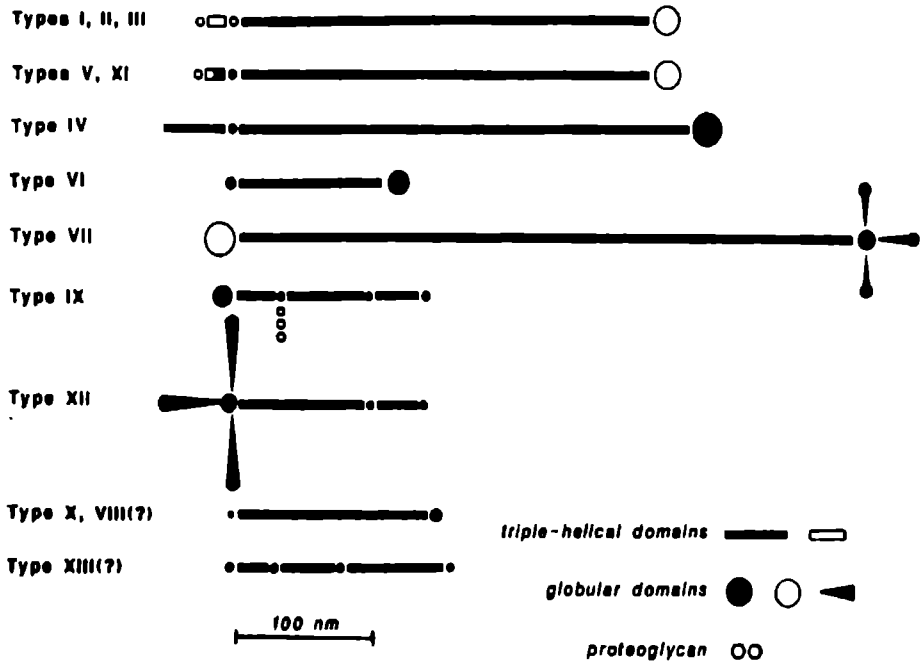

Figure 3: Molecular structure of the collagen types. Collagens contain triple-helical (solid and open rods) and globular domains (open and filled circles). Portions of the initially synthesized molecules are removed prior to their incorporation into insoluble matrices (open rods and circles) and the rest of the molecule remains intact in the matrix (closed circles and rods). The domains and their distribution are drawn approximately to scale. From: Burgeson RE and Nimni ME. Collagen types: Molecular structure and tissue distribution. Clin. Orthopaedics 282: 250-272, 1992 (with permission)

collagen type IV, which is specific for basement membranes, were the first to be identified and their complex biosynthesis and structure have been elucidated to a large extent.

COLLAGEN STRUCTURE

The basic structure of collagen as a protein with three polypeptide chains has been established in the late nineteen-fifties. All known collagens contain two distinct

types of structural domains: the triple helical domain and the globular domains. Three lefthanded helical polypeptide chains, called α-chains, each of which comprises about 1,000 amino acid residues, are coiled around each other in a stiff right-handed supercoil. The amino acid sequence of the polypeptide chains is unique in that every third residue is glycine and that it possesses the regularly repeating tripeptide Gly-X-Y. With very few exceptions proline (Pro) is always present in the X position and hydroxyproline (Hyp) prevails in the Y position. The Gly-Pro-Hyp sequence makes up about 10% of the entire molecule [42,43].

The helix comprises one complete turn for about every three residues. This conformation is made possible by the absence of side chains on the glycine residues. The hydroxyl groups of the hydroxyproline residues form strong interchain hydrogen bonds thus stabilizing the triple helix.

Hydroxyproline and hydroxylated lysine residues, hydroxylysine, are relatively specific to collagen. Glycosylation of the collagen molecules is exclusively through the hydroxylysine residues. Short non-helical peptide sequences, called telopeptides, at the NH2- an COOH-terminal ends are characteristic for the α-chains of native collagen molecules. The telopeptides are regarded to be essential for the formation of inter-chain and intermolecular crosslinks.

COLLAGEN BIOSYNTHESIS

The biosynthesis of (pro)collagen proceeds according to the general principles of synthesis of secreted proteins. The collagens are synthesized as precursor forms or procollagens containing N- and C-terminal extensions on their peptide chains. A simplified scheme for the biosynthesis of interstitial collagens is indicated in Figure 4. While elongation of the amino acid chains is still in progress, prolyl and lysyl residues are hydroxylated and hydroxylysyl residues become glycosylated. Hydroxylation of proline and lysine residues is quite unique for collagen proteins. The extent of these modifications are mediated by the primary structure of the peptide backbone which

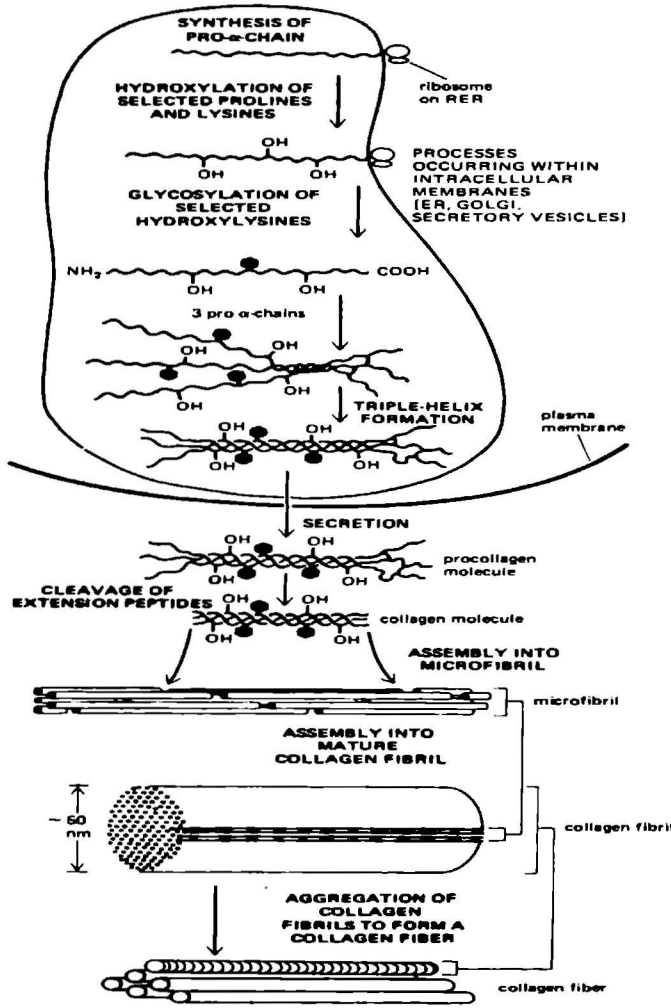

Figure 4: Schematic drawing of the various intracellular and extracellular events involved in the formation of the collagen fibril. From: Chapter 12, pp 673-715, Cell-cell adhesion and the extracellular matrix. In: The molecular biology of the cell. Alberts B., Bray D., Lewis J., Raff M., Roberts K. and Watson J.D. (eds.). Garland Publishing Inc. New York and London, 1983. (with permission)

determines the folding of the peptide chains during synthesis. It thus controls post-translational processes by

limiting the access of modifying enzymes to only a few specific sites. Hydroxylation and glycosylation can only take place before the triple helix is formed. Between cysteine residues present in both the NH2-terminal and COOH-terminal extensions interchain disulphide bonds are formed. Disulphide bond formation in the telopeptides is thought to guide the chain assembly and final folding of the triple helical procollagen molecules. After triple-helix formation is completed within the cisternae of the endoplasmatic reticulum, the procollagens are rapidly translocated through the Golgi-complex towards the plasma membrane. Subsequently, the newly synthesized procollagen molecules are released into the extracellular space where they encounter a number of processing enzymes and undergo fibril formation and crosslinking [43].

The extracellular procollagens are processed to collagen by the partial removal of the NH2- and COOH-terminal extensions. The enzymes are procollagen N-proteinases and procollagen C-proteinases and lysyl oxidase. All the procollagen proteinases isolated so far are neutral, calcium-dependent metalloproteinases [44]. The N-terminal extensions are removed prior to the C-terminal extensions. The intermediate procollagen without a NH2-propeptide is referred to as Pc-collagen. Consistently, Pn-collagens (procollagen molecules with a removed COOH extension, but with an intact NH2 extension) are rarely observed. Pn-collagens are occasionally reported as a result of a genetic disorder, which has been shown to have an impact on collagen biosynthesis e.g. in dermatosparaxis and osteogenesis imperfecta [45-48].

Lysyl oxidase initiates cross-linking in collagens by oxidative deamination of certain lysine and hydroxylysine residues located in the short N- and C-terminal non-triple helical regions (called telopeptides) [49]. Under physiological conditions the resultant triple-helical molecules, called tropocollagens, are resistant to further degradation by proteases other than specific collagenases. The tropocollagen molecules spontaneously assemble into a precisely arranged packing pattern, the collagen fibril. Initially, collagen fibrillogenesis is a reversible process but the formation of covalent crosslinks within and between

constituent tropocollagens gradually strengthens the newly formed fibrils.

The biosynthesis of the collagen α-chains, their intra- and extracellular modifications and the final assembly of tropocollagen into fibrils requires the activity of a wide spectrum of enzymes. Inappropriate functioning of these enzymes, either caused by a structurally changed target site on the (pro)-collagen molecules, or by a deficiency of an enzyme or one of its cofactors, have been incriminated in many "collagen-associated diseases". A structural defect in the amino terminus of the pro-α1(I) chain of type I procollagen, resulting in the loss of the N-proteinase cleavage site, was shown to be responsible for the type VII form of the Ehlers-Danlos syndrome [50]. In scurvy, a human disease caused by a dietary deficiency of ascorbic acid (vitamin C is one of the cofactors of the enzyme prolyl-hydroxylase), the insufficient hydroxylation of proline inhibits procollagen helix formation as a result of which the skin and blood vessels become extremely fragile [51]. Single base changes in the codons for glycine account for most of the mutations, which reflects the strict stereochemical requirement for glycine at every third position in the collagen triple helix [45]. Genetic disorders of collagen biosynthesis usually affect tissues in which the proper development and integrity of connective tissues is of paramount importance. Hence, the majority of these disorders affect bone (osteogenesis imperfecta), skin and joints (Ehlers-Danlos syndrome), arteries (Marfan syndrome) and cartilage development. Comprehensive information on a number of genetic disorders affecting collagen proteins can be found in references [45,52,53].

DEGRADATION OF COLLAGENS

Regulation of collagen homeostasis is not only confined to its biosynthetic pathway but is also controlled by, inhibitor modulated, enzymatic degradation. Degradation of extracellular matrix components is a prerequisite action in many normal physiological processes. When a tissue undergoes rapid remodelling, as observed in early development of long bones, in the postpartum uterus or in wound healing where

abrupt physiological changes in tissue mass occur, the rate of collagen removal is rapid and extensive [54].

Two important pathways of collagen breakdown have been studied in detail during the last decades: an intracellular one and an extracellular one [55,56]. *In vivo*, both pathways might be simultaneously active and complementary to each other. However, intracellular degradation of collagen appears to be restricted to physiological turnover and remodelling [57-59], while extracellular breakdown is more frequently observed under pathological conditions as in rheumatoid arthritis [60-62] and during tumor cell invasion [63,64].

INTRACELLULAR DEGRADATION OF COLLAGEN

Intracellularly degraded collagen has been shown to be of both intra- and extra-cellular origin. Evidence for the "breakdown-before-secretion" concept of newly synthesized procollagen molecules have been provided by time-course experiments of Laurent and co-workers [57,59] and Bienkowski and colleagues [65,66]. Berg and co-workers [67] demonstrated that cultured lung fibroblasts induced to synthesize abnormal procollagen, were capable of shunting a portion (up to 18 per cent) of such procollagen to their lysosomes for intracellular digestion.

The intracellular degradation of collagen from the extracellular space, necessitates the phagocytosis of collagen fibrils by connective tissue cells. It has been suggested, that prior to their uptake these fibrils are somewhat digested by extracellular collagenolytic enzymes [68,69]. However, Everts et al. [70] demonstrated a mammalian collagenase-independent uptake of collagen fibrils in cultured periosteal explants.

Both phagocytosed fibrils and non-secreted defective procollagen molecules are digested in the lysosomal apparatus. The existence of a lysosomal route of collagenolysis was supported by Etherington [71,72] and Burleigh et al. [73] who demonstrated the degradation of both soluble and insoluble collagen by the lysosomal enzymes cathepsin B and collagenolytic cathepsin. More recently, Kirschke and Barrett [74] showed that two other lysosomal

enzymes, the cysteine-proteinases cathepsin L and N, are potent collagenolytic enzymes. All the enzymes mentioned here only function at rather acid Ph which limits their activity to the small, acidified, intracellular compartments of phagocytic cells. However ,there is some evidence that a specially generated acidic environment outside the cell, e.g. at the membrane/fibre interface, permits some of the acid proteinases to attack extracellular collagen fibrils [75]. Local pericellular acidification down to pH values where these enzymes can operate has recently been demonstrated [76-78].

EXTRACELLULAR DEGRADATION OF COLLAGEN

The irreversible extracellular breakdown of connective tissues and especially its collagenous component has been subject of extensive research during the last decades. The supposed role that collagenolytic enzymes may play in many inflammatory diseases, in wound healing and tumour invasion and metastasis undoubtedly has had its impact on its current scientific attention [79-81].

The enzymatic mechanisms whereby collagen is degraded, other than by the attack of bacterial collagenases, generally involves the cooperative action of a number of enzymes. *In vitro*, collagen has been shown to be degraded as a whole, or in part, by enzymes representing all four classes of proteinases. Several degradation schemes have been proposed which include at least three or four different steps [82,83].
In general, we can distinguish the following steps:

1) depolymerization of collagen fibres
2) physiological collagenolysis
3) further extracellular breakdown of collagen-derived peptides.

1] *DEPOLYMERIZATION*

Prior to the action of true mammalian collagenases (enzymes which attack the helical core of native collagen molecules under physiological conditions) a depolymerisation

of the polymeric collagen fibres is considered necessary [84]. Two mechanisms of depolymerization have been suggested. The first proposes the cleavage of the cross-links in the telopeptides [85,86]. A second mechanism assumes the existence of a proteolytic attack on the non-helical carboxyterminal telopeptide between the helix and

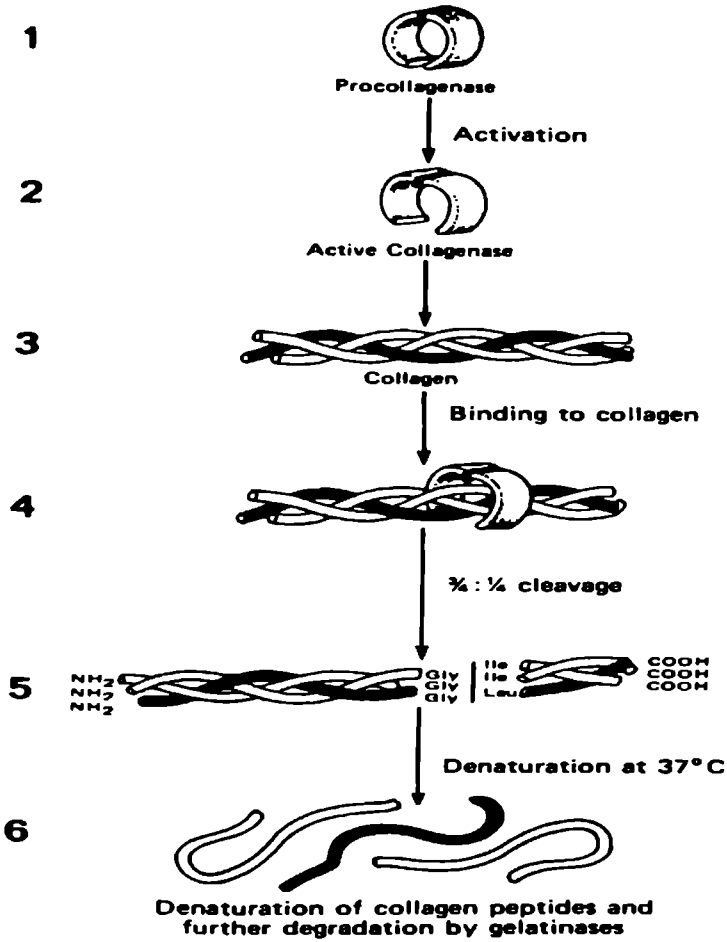

Figure 5: Procollagenase activation and its interaction with collagen. Modified from: Mignatti P., Welgus H.G. and Rifkin D.B. Role of degradative enzymes in wound healing. Chapter 21, pp 497-524. In: The Molecular and Cellular Biology of Wound Repair. Clark R.A.F. and Henson P.M.(eds). Plenum Publishing Coop. New York, 1988. (with permission)

its nearest lysyl residue, which is known to be important in the formation of intermolecular cross-links [87,88]. For both mechanisms candidate enzymes have been proposed. Starkey et al. [89,90] suggested that both elastase and cathepsin G derived from polymorphonuclear leucocytes could destabilize collagen fibrils. The leucocyte enzymes are thought to play a substantial role in collagen destruction in inflamed connective tissues [91,92].

A telopeptidase, with all the characteristics of a metallo- proteinase, described by Goldberg and Scott [88] was isolated from porcine gingiva explants and shown to be active under similar conditions as interstitial collagenase.

Although a partial solubilization of the collagen molecules at the surface of the fibrils certainly would facilitate the action of a true collagenase by increasing its susceptibility, it is not yet clear if this is merely a supportive or an elementary step in collagen degradation.

2] *PHYSIOLOGICAL COLLAGENOLYSIS*

The subsequent, crucial, step in the proposed collagen degradation scheme is the hydrolysis of the triple helix . A schematic drawing of the extracellular degradation of collagen by mammalian collagenases is given in Figure 5. The triple helical structure of native fibrillar collagens (Types I, II and III) is extremely resistant to the action of most digestive proteases. However, a closely related group of enzymes, the vertebrate collagenases, do attack the helical core at one very specific locus - a point approximately three-quarters of the length of the molecule from its amino-terminal end. The cleavage products, designated TCa (75%) and TCb (25%), can be separated electrophoretically from their native α-chains and are regarded as the ultimate evidence for the presence of a true mammalian collagenase.

Essentially all of the known mammalian collagenases appear to cleave the collagen helix at the same locus, i.e, Gly-Ile in the α1 chain of type I collagen and Gly-Leu in the α2 chain (positions 775-776 in the amino acid sequence) [93]. The first enzyme described with such a unique specificity was isolated from the culture medium of tadpole

tail tissue [94]. Since its first detection similar enzymes have been isolated from the culture medium of a large number of cells and tissues obtained from a variety of species. The cells include fibroblasts, keratinocytes, macrophages, polymorphonuclear leucocytes and tumour cells [95-99]. Recent data suggest that there are two immunologically and kinetically distinct interstitial collagenases, one secreted by connective tissue cells and macrophages and another stored exclusively in PMN-leucocyte granules, which are released during degranulation [98-100].

3] *EXTRACELLULAR BREAKDOWN OF COLLAGEN-DERIVED PEPTIDES*

The fragments formed by the action of collagenase, comprising the amino-terminal three-quarter and the carboxy-terminal one-quarter of the molecule dissociate from the fibril, spontaneously lose their helical conformation *in vitro* at 37°C and denature to gelatin. The structurally less complex gelatin chains are susceptible to degradation by less specific proteases. Although very highly purified collagenases can degrade gelatins at a low rate, gelatin is generally regarded as a relatively poor substrate for interstitial collagenase [101].

A specific group of enzymes, present in the extracellular space, where they are synthesized by a variety of connective tissue cells [102], and in secretory granules of neutrophils [103], has been demonstrated to preferentially degrade gelatin at neutral pH and collectively has been referred to as gelatinases. Subsequently, both the tissue and PMN-leucocyte gelatinases were demonstrated also to degrade native and soluble type IV and V collagen [104-106].

Recent studies have shown the existence of two immunologically distinct gelatinases, with very similar substrate specificities migrating at either a molecular weight of approximately 72 kDa or 92 Kda in SDS-PAGE [107,108]. The high molecular weight, glycosylated, gelatinases are secreted by macrophages, monocytes and neutrophils and by some tumour cells. Connective tissue cells predominantly secrete the lower, mainly non-glycosylated, molecular weight species. A type IV collagen degrading enzyme from tumour cells and transformed

connective tissue cells [109], referred to as type IV collagenase, was reported to be immunologically related to the gelatinases produced by circulating cells [110]. Sequence homology between the type IV collagenase and the low M_r gelatinase of connective tissue cells was demonstrated by Collier et al. [111]. Latter studies have further strengthened the hypothesis that all the gelatinases and type IV collagenases described to date are closely related proteinases which merely differ in their degree of glycosylation.

The small fragments that remain after gelatinase action are further degraded both extracellularly by peptidases as well as intracellularly in the catheptic phago-lysosomal pathway.

DEGRADATION OF BASEMENT MEMBRANE COLLAGEN TYPE IV

The basement membranes collagenous matrices separate the parenchymal cells of various tissues from underlying interstitial stroma. Basement membranes limit the penetration of macromolecules through blood vessel walls, act as a scaffold for epithelial cells and promote cell differentiation [35,38,112].

The major collagenous component of basement membranes is type IV collagen and is distinct from the other types as judged by location and chemical and immunological criteria. Collagen type IV is not attacked by the interstitial collagenases [112]. This basement membrane collagen type is degraded by an altogether different set of proteases. A type IV collagen degrading enzyme was first isolated from the culture medium of a highly metastatic mouse tumour explant by Liotta and colleagues [113]. The type IV collagenase described by this group cleaved the type IV collagen molecule within the helical domain but failed to degrade type V collagen. Type IV collagen molecules are cleaved in a similar fashion as the interstitial collagens generating a three-quarter C terminal and a one-quarter N-terminal fragment [114]. Now, most of the gelatinolytic enzymes purified from mesenchymal cells, tumour cells, macrophages and PMN-leucocytes have been assessed for their potential

activity against type IV collagen. From these studies it became clear that the 72 Kda gelatinase and type IV collagenase activity is essentially the activity of a single enzyme exhibiting minor inter-tissue and species differences [98,99,115].

Names	Deduced Mass (kDa)	Nr of Domains	ECM Substrates
COLLAGENASES			
Interstitial collagenase (EC 3.4.24.7) MMP-1, fibroblast collagenase	54.1	4	Collagen types I, II, III, VII and X
Neutrophil collagenase (EC 3.4.24.34)	53.4	4	Collagen types I, III and III
GELATINASES			
Gelatinase A (EC 3.4.24.24) MMP-2, 72 kD gelatinase type IV collagenase	73.9	5	Gelatins, collagen IV ?, V and elastin
Gelatinase B (EC 3.4.24.35) MMP-9, 92 kD gelatinase, 92 kD type IV collagenase	78.4	6	Gelatins, collagen IV?, V and elastin
STROMELYSINS Stromelysin-1 (EC 3.4.24.17) MMP-3, transin, proteoglycanase procollagenase activator	54.0	4	Proteoglycans, laminin, fibronectin, gelatins, collagens III, IV, V, IX
Stromelysin-2 (EC 3.4.24.22) MMP-10, transin-2	54.1	4	Proteoglycans, laminin, fibronectin, gelatins, collagens III, IV, V, IX
Matrilysin (EC 3.4.24.23) MMP-7, Pump-1, uterine metalloproteinase	29.7	3	Proteoglycans, gelatins collagen IV?, elastin, fibronectin
Others			
stromelysin-3	54.6	4	?
metalloelastase	53.9	4	elastin, fibronectin

Table I: Characteristics of the Matrix Metalloproteinase family members. From: Matrisian L.M. The matrix-degrading metalloproteinases. BioEssays 14: 455-463, 1992. (with permission)

THE MATRIX METALLOPROTEINASES

Both the interstitial collagenases and 72 Kda gelatinase A (type IV collagenase) are endopeptidases and have been classified as matrix metalloproteinases. This group of

proteinases also includes the enzyme stromelysin-1 (originally called proteoglycanase [98,99,116] and at least two other metalloproteinases with a strong preference for extracellular matrix proteins as substrates. Thus, the matrix metalloproteinases (also referred to as matrixins or MMP's) are generally considered to be key enzymes in extracellular matrix turnover [115,117,118]. Stromelysin-1 is a particularly remarkable enzyme in that it is active over a wide pH range (5.0 - 9.0) and degrades a broad range of substrates. This proteinase is now regarded as one of the most important catabolic enzymes of the ECM, synthesized and secreted by the very same connective tissue cells that produce the matrix.

The properties of the three major MMP subclasses - collagenases, gelatinases and the stromelysins - vary little with species and cell type; all appear to cleave their collagenous substrates at a Gly-Leu or Gly-Ile peptide bond. Characteristics common to most of the matrix metalloproteinases are presented in Table I.

The recent elucidation of the primary amino acid sequences of rabbit synovial collagenase [119], human fibroblast collagenase [120,121], human stromelysin-1 [122] and stromelysin-3 [123] and the rat stromelysin homologues transin and transin-2 [124,125] revealed extensive sequence homologies between these enzymes and confirmed their proposed relationship [126]. Conserved features include two cysteine residues present in the active form of the enzymes, a zinc chelation site and two potential glycosylation sites. In gelatinase A the catalytic domain is split by a fibronectin binding domain, while gelatinase B has an additional collagen binding domain.

THE BIOLOGICAL REGULATION OF METALLOPROTEINASE ACTIVITIES.

Modulation of extracellular matrix degradation can take place by control of the synthesis and secretion of metalloproteinases, by control of the factors responsible for activation of the latent metalloproteinases, by control of the amount of inhibitor(s) synthesized and secreted and by control of the removal of the metalloproteinases and/or inhibitors in the extracellular space. Figure 6 summarizes

the main events associated with the proteolytic degradation of the extracellular matrix.

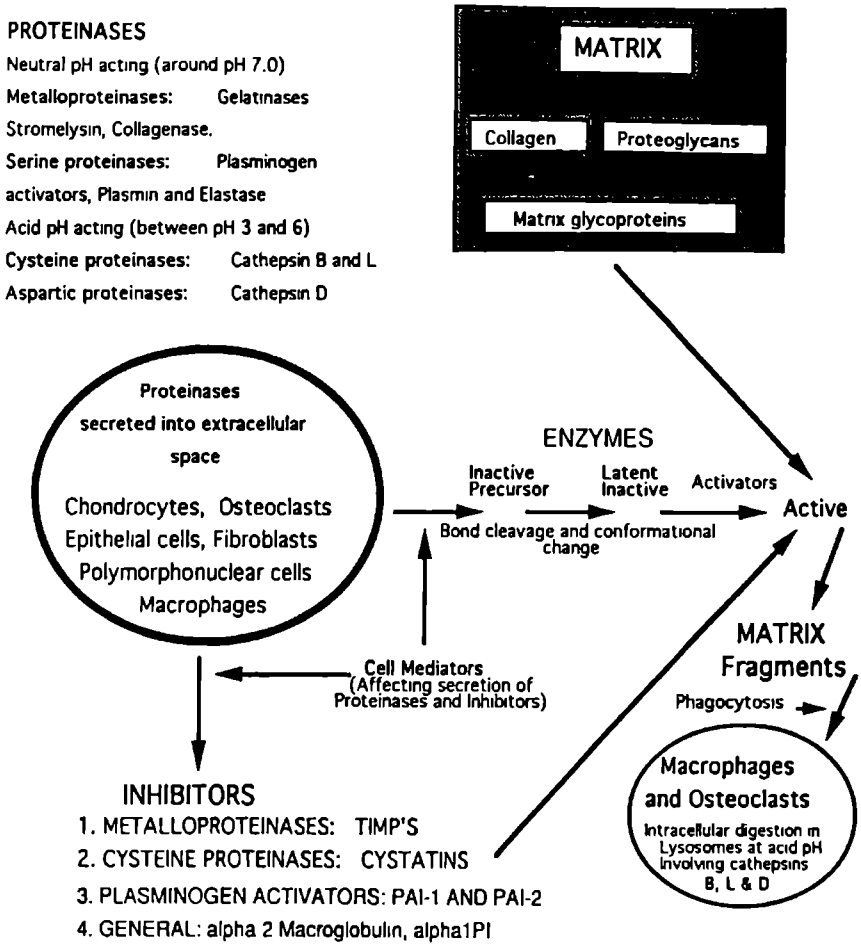

Figure 6: Schematic drawing of the secretion, activation and inhibition of proteinases potentially involved in the degradation of the extracellular matrix.

REGULATION OF MATRIX METALLOPROTEINASE EXPRESSION

A significant feature of cells secreting metalloproteinases and their inhibitors (Tissue Inhibitor of Metalloproteinase: TIMP) is their extreme sensitivity to soluble stimuli such as hormones, growth factors, vitamins

and tumor promotors (e.g. phorbol esters). It is thought that the effect of these regulating factors on the metalloproteinases and their inhibitors determines the integrity of the extracellular matrix in both health and disease. Treatment of synovial and cartilage explants in culture with corticosteroids suppressed the production of MMP's [127,128]. Burnett et al. [129] reported, a significant increase of TIMP sputum levels in patients with chronic bronchitis after treatment with prednisolone. Progesterone was shown to regulate the activity of collagenase and the gelatinases A and B in human endometrial explants [130]. Matsusue and Walser [131], using adrenalectomized rats, showed that corticosterone plasma levels are to be kept within confined limits in order to accomplish proper intestinal anastomotic healing.
Corticosteroids, shown to be capable of regulating both MMP and TIMP production might thus be important in matrix remodelling as observed in diseased joints and during wound healing.

A number of reports have shown that the cytokine interleukin-1ß (IL-1ß) is a very potent inducer of MMP-1 (collagenase) and/or MMP-3 (stromelysin) [132-135]. Although IL-1ß did not alter the expression of the 72kD gelatinase (MMP-2) [136] it did induce the 92kD form (MMP-9) in synovial fibroblasts allowing these cells to degrade MMP-1 and MMP-3 resistant interstitial elements such as collagen types IV, V and XI [133]. Meikle et al. [137,138] showed that the cytokines IL-1 and tumor necrosis factor α (TNF-α), released from circulating mononuclear cells, could induce collagenolysis of periodontal tissues in a dose-dependent manner while total TIMP levels remained unchanged. *In vivo* production of these cytokines is believed to be induced by bacterial antigens.

The cellular response to IL-1ß was subsequently shown to be either up- or down-modulated by other growth factors. The platelet derived growth factor (PDGF), which was also found to be synthesized and released from activated macrophages [139], exhibited a synergistical response towards IL-1ß induction of MMP-1 and MMP-3 [140]. On the other hand it was demonstrated by several groups that transforming growth factor-beta (TGF-ß) can control the effects of several other

growth factors such as IL-1ß, IL-2, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [141-143] on MMP secretion. TGF-ß, a stimulator of the synthesis and deposition of extracellular matrix components, may perform this function by selectively repressing the IL-1 induction of MMP's by reducing the number of IL-1 receptors [132] but also by simultaneously enhancing the expression of TIMP [143,144]. In addition, TGF-ß can directly exert its effect on the synthesis of new collagen by regulating a gene crucial for the production of this molecule [145].

Another representative of the interleukin family, IL-6, did not modify MMP-1 expression but was shown to induce the synthesis of TIMP-1 in a number of connective tissue cells [146]. The cytokine Il-4 caused a dose-dependent suppression of macrophage 92-kD gelatinase (MMP-9) production without affecting TIMP levels [147]. At the same time it was shown that IL-4 blocked the synthesis of MMP-9 by human monocytes via a prostaglandin-E2 linked mechanism [148].

TNF-α and EGF enhanced the steady state levels of the messenger for MMP-1 [149] but were also found to act at the protein level since an increase in the secretion of this enzyme was observed [150]. The binding of TNF-α to high affinity receptors profoundly affected gene regulation and stimulated e.g. the IL-1 and IL-6 genes [151] an effect not unexpected from the 'boss' of the cytokines.

ACTIVATION OF METALLOPROTEINASE PROENZYMES

Normal levels of metalloproteinase activities in unstimulated cell or explant culture systems are below or near the detection limit of activity-based assays. This finding is due to their secretion as latent enzymes and their instant capture by inhibitors present in serum.

Activation is thought to be a key point in the regulation of matrix metalloproteinase activity and has been the subject of several studies. *In vitro* activation of metalloproteinases derived from connective tissue cells can be accomplished by addition of organomercurials, such as p-Aminophenylmercuric acetate (APMA) and p-Chloromercuric-benzoic acid (pCMB), by surfactants such as sodium dodecylsulphate (SDS) or by disulfide compounds such as

oxidized glutathione. These modes of activation of latent collagenase involve the dissociation of cysteine residue 73 (Cys^{73}) from the active-site zinc atom and its replacement by water. This process is referred to as the "cysteine switch", with the concomitant exposure of the active site (Figure 7) [152,153]. Others support the theory that activation occurs by means of a stepwise loss through proteolysis of an Mr 9000 propeptide from the N-terminus of the native molecule [154,155]. One of the later steps in this cascade was shown to be autocatalytic. Conformational perturbations induced under denaturing conditions (APMA, low pH, SDS and an increased temperature) might disrupt the propeptide functions and thus be sufficient to activate.

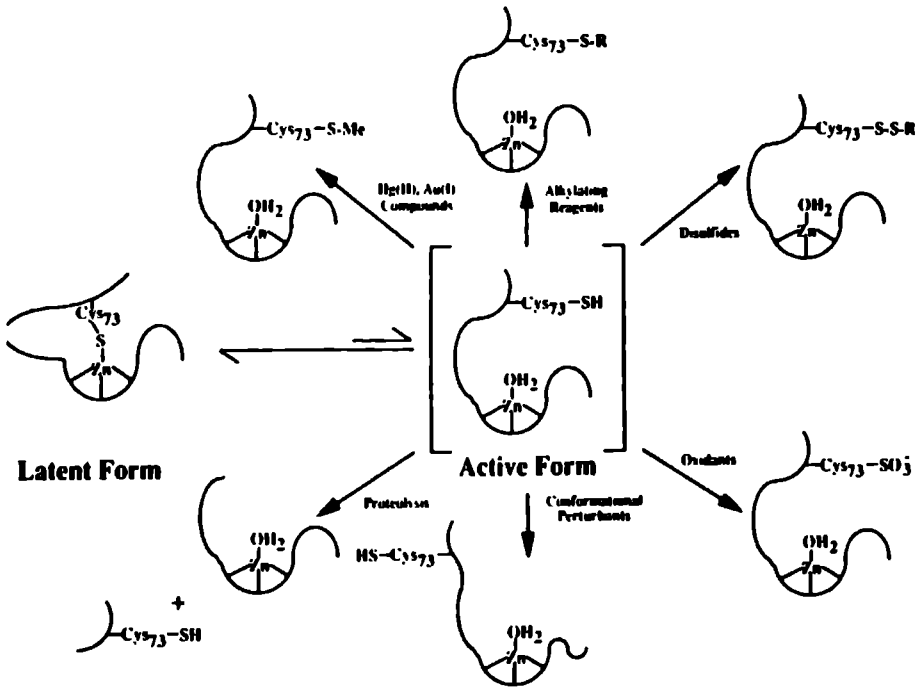

Figure 7: Model describing the underlying biochemical basis for the multiple modes of activation of latent human fibroblast collagenase. From: Springman et al. Multiple modes of activation of latent human fibroblast collagenase: Evidence for the role of a Cys^{73} active site zinc complex in latency and a cysteine switch mechanism for activation. Proc. Natl. Acad. Sci. USA 87: 364-368, 1990. (with permission)

The mechanisms underlying the *in vivo* activation of these enzymes are still poorly understood. Limited proteolysis by trypsin, plasmin, neutrophil elastase and cathepsin G are established methods to activate metalloproteinases in a test tube [156]. Unlike collagenase and stromelysin, gelatinase is poorly activated by exogenous proteinase action [102-104]. A mechanism for which some experimental support has been obtained is the involvement of a proteolytic cascade, similar to blood coagulation [157]. An important step in the *in vivo* activation of metalloproteinases might be the generation of plasmin from its zymogen plasminogen by plasminogen activators [158-160]. Plasminogen activation possibly involves the cysteine proteinases cathepsin B and L [161,162]. Furthermore, it appears that metalloproteinases can activate each other: recently stromelysin was shown to significantly enhance the activity of an MMP-1 preparation [163,164].

INHIBITION OF METALLOPROTEINASES

Metalloproteinases are subject to inactivation by the serum inhibitor α2-macroglobulin and an expanding family of tissue inhibitors of metalloproteinases (TIMPs) [165]. Activated collagenase is inactivated by formation of a 1:1 complex with the tissue inhibitor of metalloproteinases. TIMP is a heat stable sialoglycoprotein of approximately 28 Kda and contains six disulfide linkages [166]. TIMP can be destroyed by reduction and alkylation [166], lacks species specificity but does not inhibit bacterial collagenases. The inhibitory activity of TIMP against MMP-1, 2, and 3 was almost completely abolished by reacting the inhibitor with either trypsin, α-chymotrypsin or PMN-leucocyte elastase. Cathepsin G and pancreatic elastase showed less degradative effects on TIMP [167]. A non-glycosylated inhibitor, IMP or TIMP-2 was later isolated from a complex with the 72 Kda progelatinase A [168]. However, the complex of MMP-2 with TIMP-2 does not inhibit its gelatinolytic activity suggesting that binding is distant from the active centre of the enzyme [169]. TIMP-2 was shown to be capable of inhibiting the autocatalysis of gelatinase A but another molecule of either TIMP or TIMP-2 was required to inhibit

gelatinolysis [170]. A third member of the family (ChIMP-3) exclusively located in the matrix was recently isolated from chicken embryo fibroblasts [171].

The ability of TIMPs to inhibit not only collagenase but other MMP's as well suggests an important role in the regulation of ECM degradation. TIMP synthesis and secretion appears to be executed by the same cells that are known to secrete metalloproteinases thus requiring a tightly controlled coordination.

AIM OF THE STUDY

The understanding of the balance between synthesis and degradation of collagen in the healing intestine has been one of the long term objectives of the research undertaken in the Department of General Surgery. Indirect evidence suggests that an imbalance between a collagenolytic enzyme and its natural inhibitor might be causative to disturbed anastomotic repair. However, experimental data on collagenolytic enzyme activities in intestinal wounds are lacking. Therefore, we have investigated the putative role in wound healing of an essential enzyme in the degradation of collagen, the metalloproteinase collagenase. To this end methods were developed to extract and assay enzymatic activity present in experimental anastomoses, using the rat as a model. Anastomoses were constructed simultaneously in both ileum and colon so that we would be able to correlate our data with the clinical observation that healing of colonic wounds on average poses more problems than an ileal wound. Furthermore, we have attempted to purify the enzyme collagenase (MMP-1) from the culture medium of rat and human derived skin fibroblasts. This work was started in order to obtain pure enzymes for subsequent immunohistochemical studies. In addition, a comparison between the rat and human enzyme was thought to be useful in the interpretation of data obtained from our rat model and in extrapolating to the human situation.

Since a large number of colorectal anastomoses in patients is constructed following the excision of neoplastic growth and the processes of wound healing and tumor cell invasion show remarkable similarities, we have also investigated the activity of collagenase in human tumour specimen and matched non-invaded mucosal tissue obtained at surgery. Collagenolytic activities were correlated with both clinico-pathological stage and grade of histological differentiation.

REFERENCES

1 **Haddow A.** Molecular repair, wound healing and carcinogenesis: tumor production a possible overhealing? Adv. Cancer Res. 16: 181-234, 1972

2 **Dvorak HF.** Seminars in Medicine : Tumours: Wounds that do not heal. Similarities between tumour stroma generation and wound healing. New England J. Med. 315/26: 1650-1569, 1986

3 **Whalen GF.** Solid tumours and wounds: transformed cells misunderstood as injured tissue? The Lancet 336: 1489-1492, 1990

4 **Sporn MB, Roberts AB.** Peptide growth factors and inflammation, tissue repair and cancer. J. Clin. Invest. 78: 329-332, 1986.

5 **Sporn MB, Roberts AB.** Autocrine secretion : 10 years later. Annals of Internal Medicine 115/5: 408-414, 1992

6 **Grillo HC and Gross J.** Collagenolytic activity during mammalian wound repair. Develop Biol 15: 300-317,1967.

7 **Donoff RB, McLennan JE, Grillo HC.** Preparation and properties of collagenases from epithelium and mesenchyme of healing mammalian wounds. Biochim. Biophys. Acta 227: 639-653,1971.

8 **Woessner JF, Dannenberg AM, Pula PJ, Selzer MG, Ruppert CL, Higuchi K, Kajiki A, Nakamura M, Dahms NM, Kerr JS, Hart GW.** Extracellular collagenase, proteoglycanase and products of their activity, released in organ culture by intact dermal inflammatory lesions produced by sulfur mustard. J. Invest. Dermatol. 95: 717-726, 1990.

9 **Hastings C, van Winkle W, Barker E, Hinds J, Nichols W.** Effect of suture material on the healing wounds of stomach and colon. Surg. Gynecol. Obstet. 140: 701-707, 1975

10 **Martens MFWC, Huyben CMLC, Hendriks Th.** Collagen synthesis in fibroblasts from human colon: Regulatory aspects and differences with skin fibroblasts. Gut 33: 1664-1670, 1992.

11 **Hendriks Th, Mastboom W** Healing of experimental intestinal anastomoses: parameters of repair. Dis. Colon Rectum 33: 891-901, 1990.

12 **Turunen MJ, Peltokalio P.** Surgical results in 657 patients with colorectal cancer. Dis. Colon Rectum 26: 606-612 1983.

13 **Khoury GA and Waxman BP**: Large bowel anastomoses. I. The healing process and sutured anastomoses. A review. Br. J. Surg. 70: 61- 63, 1983

14 **Koruda MJ and Rolandelli, RH.** Experimental studies on the healing of colonic anastomoses. J. Surg. Res. 48: 504-515 1990.

15 **Hesp WLEM, Lubbers EJC, De Boer HHM, Hendriks Th.** Anastomotic insufficiency in small bowel surgery - incidence and treatment

Langenbecks Arch. Chir. 368: 105-111, 1986
16 **Fielding LP, Stewart-Brown S, Blesovsky L, Kearney G.** Anastomotic integrity after operations for large bowel cancer. A multicentre study. Br. Med. J. 281: 411-414, 1980
17 **Shoshan S.** Wound healing Int. Rev. Connect. Tissue 9: 1-26, 1981
18 **Komura T.** The lattice arrangement of the rat small intestine: scanning electron microscopy. Cell Tissue Res. 251: 117-121, 1988.
19 **Hunt TK, Hawley PR, Hale J, Goodson W, Tharkal KK.** Colon repair: the collagenous equilibrium. In: Wound healing and wound infection. Theory and surgical practice, TK Hunt (ed.) Appleton-Century-Crofts New York, pp 153-159, 1980
20 **Eckersley JRT, Dudley HAF.** Wounds and wound healing. Br. Med. Bull. 44: 423-436, 1988.
21 **Martens MFWC, Hendriks Th.** Postoperative changes in collagen synthesis in intestinal anastomoses of the rat. Gut 32: 1482-1487, 1991.
22 **Shike M, Winawer SJ, Greenwald PH, Bloch A, Hill MJ, Swaroop SV.** Primary prevention of colorectal cancer. The WHO collaborating centre for the prevention of colorectal cancer. Bull. World Health Organ. 66: 377-385, 1990
23 **Lynch HT, Smyrk T, Watson P, Lanspa SJ, Boman BM, Lynch PM, Lynch JF, Cavalieri J.** Hereditary colorectal cancer. Seminars in Oncology 18/4: 337-366, 1991
24 **Muto T, Bussey HJR, Morson BC.** The evolution of cancer of the colon and rectum. Cancer (Phila.) 36: 2251-2270, 1975
25 **Fearon ER, Vogelstein B.** A genetic model for colorectal tumorigenesis. Cell 61: 759-767, 1990
26 **Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, van Tuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R and Vogelstein B.** Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244: 217-221, 1989
27 **Paraskeva C, Finerty S, Harper S, Williams AC.** Cellular and Molecular events involved in tumour progression in colorectal carcinogenesis. A study of the adenoma to carcinoma sequence *in vitro* In: Hereditary Colorectal Cancer (Utsunomiya, J and Lynch HT eds.) pp 497-507, 1990
28 **Liotta LA, Garbisa S, Tryggvason K.** Biochemical mechanisms involved in tumor cell penetration of the basement membrane. In: Liotta LA, Hart IR (eds.), Tumor invasion and metastasis. Martinus Nijhoff, Boston, pp 319-336, 1984
29 **Liotta LA, Rao CN, Wewer UM.** Biochemical interactions of tumor cells with the basement membrane. Ann. Rev. Biochem. 55: 1037-1057, 1986

30 **Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer J, Kronberger A, He CH, Bauer EA, and Goldberg GI.** H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. J. Biol. Chem. 263/14: 6579-6587, 1988

31 **Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, Yamashita K, Hayakawa T.** Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase from HT 1080 human fibrosarcoma cells. J. Biol. Chem. 267/30: 21712-21719, 1992.

32 **Emonard HP, Remacle AG, Noel AC, Grimaud J-A, Stetler-Stevenson WG, Foidart J-M.** Tumor cell surface-associated binding site for the Mr 72000 type IV collagenase. Cancer Res. 52: 5845-5848, 1992.

33 **Blasi F, Vassalli J-D, Dano K.** Urokinase-type plasminogen activator: proenzyme, receptor and inhibitors. J. Cell. Biol. 104: 801-805, 1987.

34 **Mareel MM, van Roy FM, De Baetselier P.** The invasive phenotypes. Cancer and Metastasis Rev. 9: 45-62, 1990.

35 **Paulsson M.** Basement membrane proteins - structure, assembly and cellular interactions. Critical Reviews in Biochemistry and Molecular Biology 27 (1-2): 93-127, 1992.

36 **Cunningham LW** (edt.). Structural and Contractile Proteins part D: Extracellular Matrix: Chapters B, C and D. Methods in Enzymology Vol. 144 Academic Press, Inc., 1987.

37 **Engel J.** Laminins and other strange proteins. Biochemistry 31/44: 10643-10651, 1992.

38 **Timple R.** Review: Structure and biological activity of basement membrane proteins. Eur. J. Biochem. 180: 487-502, 1989.

39 **McDonald JA.** Fibronectin: a primitive matrix. In: The molecular and cellular biology of wound repair. (Clark RAF and Henson PM eds.) Chapter 18 pp 405-411, 1988.

40 **Scott JE.** Supramolecular organization of extracellular matrix glycosaminoglycans, *in vitro* and in the tissues. FASEB J. 6: 2639-2645, 1992.

41 **Miller EJ, Gay S.** The Collagens: An Overview and Update Methods in Enzymology 144: 3-41, 1987.

42 **Burgeson RE and Nimni ME.** Collagen types. Molecular structure and tissue distribution. Clinical Orthopaedics 282: 250-272, 1992.

43 **Van der Rest M and Garrone R.** Collagen family of proteins. FASEB J. 5: 2814-2823, 1991.

44 **Hojima Y, McKenzie J, van der Rest M, Prockop.** Type I N-proteinase from chick embryo tendons. J. Biol. Chem. 264: 11336-11345, 1989.

45 **Kuivaniemi H, Tromp G and Prockop DJ.** Mutations in collagen genes: causes of rare and some common diseases in humans. FASEB J. 5: 2052-2060, 1991

46 **Becker U, Timpl R, Helle O, Prockop DJ.** NH2-terminal extensions on skin collagen from sheep with a genetic defect in conversion of procollagen into collagen. Biochemistry 15/13: 2853-2862, 1976

47 **Mauch C, Aumailley M, Paye M, Lapiere CM, Timpl R, Krieg T.** Defective attachment of dermatosparatic fibroblasts to collagens I and IV. Exp. Cell Res. 163: 294-300, 1986

48 **Bonaventura J, Cohen-Salal L, Lasselin C, Allain JC, Maroteaux P.** Abnormal procollagen synthesis in fibroblasts from three patients of the same family with a severe form of osteogenesis imperfecta. Biochim. Biophys. Acta 889: 23-34, 1986

49 **Kagan HM, Trackman PC.** Properties and function of lysyl oxidase. Am J. Resp. Cell Mol. Biol. 5: 206-210, 1991.

50 **Cole WG, Chan D, Chambers GW, Walker ID, Bateman JF.** Deletion of 24 amino acids from the pro α1(I) chain of type I procollagen in a patient with the Ehlers-Danlos syndrome type VII. J. Biol. Chem. 261/12: 5496-5503, 1986.

51 **Spannheimer RG, Peterkofsky B.** A specific decrease in collagen synthesis in acutely fasted vitamin C supplemented guinea pigs. J. Biol. Chem. 260/7: 3955-3962, 1985.

52 **Prockop DJ, Kivirikko KI.** Heritable diseases of collagen. N. Engl. J. Med. 311/6: 376-386, 1984.

53 **Cheah KS.** Collagen genes and inherited connective tissue disease. Biochem. J. 229: 287-303, 1985.

54 **Welgus HG, Kobayashi DK, Jeffrey JJ.** The collagen substrate specificity of rat uterus collagenase. J. Biol. Chem. 258/23: 14162-14165, 1983.

55 **Murphy G, Reynolds JJ.** Current views of collagen degradation: progress towards understanding the resorption of connective tissues BioEssays 2/2: 55-60, 1985.

56 **Everts V, Hembry RM, Reynolds JJ, Beertsen W.** Metalloproteinases are not involved in the phagocytosis of collagen fibrils by fibroblasts. Matrix 9: 266-276, 1989.

57 **Laurent GJ.** Dynamic state of collagen : Pathways of collagen degradation *in vivo* and their possible role in regulation of collagen mass. Amer. J. Physiol. 252/1: C1-C9, 1987.

58 **Van Veen HA, Copius Peereboom-Stegeman JHJ.** The influence of the estrous cycle on the volume density and appearance of collagen containing vacuoles in fibroblasts of the rat uterus. Virchows Arch. 53: 23-31, 1987.

59 **McAnulty RJ, Laurent GJ.** Collagen synthesis and degradation *in vivo*: Evidence for rapid rates of collagen turnover with

extensive degradation of newly synthesized collagen in tissues of the adult rat. Collagen Rel. Res. 7: 93-104, 1987.
60 **Murphy G, Hembry RM.** Proteinases in rheumatoid arthritis. J. of Rheumatology 19(S32): 61-64, 1992.
61 **Okada Y, Gonoji Y, Nakanishi I, Nagase H, Hayakawa T.** Immunohistochemical demonstration of collagenase and gelatinase and tissue inhibitor of metalloproteinases (TIMP) in synovial lining cells of rheumatoid synovium. Virchows Arch B Cell Pathol. 59: 305-312, 1990.
62 **Welgus HG.** Stromelysin - structure and function. Progress Inflammation Res. and Therapy 35: 61-67, 1991.
63 **Sato H, Kida Y, Mai M, Endo Y, Sasaki T, Tanaka J, Seiki M.** Expression of genes encoding type IV collagen degrading metalloproteinases and tissue inhibitors of metalloproteinases in various human tumor cells. Oncogene 7(1): 77-83, 1992.
64 **Kato Y, Ogawa K, Yamamoto S, Abe S, Kishi JI, Hayakawa T.** A novel TIMP insensitive type IV collagen degrading metalloproteinase from murine metastatic sarcoma cells. FEBS letters 268/1: 39-42, 1990.
65 **Bienkowski RS, Baum BJ, Crystal RG.** Fibroblasts degrade newly synthesized collagen within the cell before secretion. Nature 276: 413-416, 1978.
66 **Bienkowski RS.** Intracellular degradation of newly synthesized collagen. Collagen Rel. Res. 4: 399-412, 1984.
67 **Berg RA, Schwartz ML, Rome LH, Crystal RG.** Lysosomal functions in the degradation of defective collagen in cultured lung fibroblasts. Biochemistry 23: 2134-2138, 1984.
68 **Reynolds JJ.** Inhibition of production and action of tissue metalloproteinases. Ann. Biol. Clin. 44: 188-194, 1986.
69 **Harris ED. jr Cartwright E.** Mammalian collagenases. In: Proteinases in mammalian cells and tissues, (Barrett, AJ ed.) Elsevier/North Holland Biomed. Press, Amsterdam, pp 249-283, 1977.
70 **Everts V, Beertsen W, Hembry RM, Reynolds JJ.** Collagenase is not involved in the phagocytosis of collagen fibrils by fibroblasts in cultured periosteal explants. In: Everts V., Phagocytosis and intracellular digestion of collagen fibrils. Thesis University of Amsterdam pp 69-82, 1987.
71 **Etherington DJ.** The purification of bovine cathepsin B1 and its mode of action on bovine collagens. Biochem. J. 137: 547-557, 1974.
72 **Etherington DJ.** The dissolution of insoluble bovine collagens by cathepsin B1, collagenolytic cathepsin and pepsin. The influence of collagen type, age and chemical purity on

susceptibility. Connect. Tissue Res. 5/3: 135-154, 1977.
73 **Burleigh MC, Barrett AJ, Lazarus GS.** Cathepsin B1: A lysosomal enzyme that degrades native collagen. Biochem. J. 137: 387-398, 1974.
74 **Kirschke H, Barrett AJ.** Cathepsin L - A lysosomal cysteine proteinase. Progr. Clin. Biol. Res. 180: 61-69, 1985.
75 **Etherington DJ, Maciewicz RA, Taylor MAJ, Wardale RJ, Silver IA, Murrills RA, Pugh D.** The role of collagen-degrading cysteine proteinase in connective tissue metabolism. In: V. Turk (ed.), Cysteine proteinases and their inhibitors, pp 269-282 W de Gruyter, Berlin 1986.
76 **Silver IA, Murrills RJ, Etherington DJ.** Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp. Cell Res. 175: 266-276, 1988.
77 **Griffiths, JR** Are cancer cells acidic? Br. J. Cancer 64: 425-427, 1991.
78 **Editorial**. Tumour pH. The Lancet 340: 342-343, 1992.
79 **Birkedal-Hansen H.** Collagenase in periodontal disease In: Collagenase in normal and pathological connective tissues. Woolley DE and Evanson JM (eds.) pp 127-140 John Wiley & Sons Chichester New York Brisbane Toronto 1980.
80 **Biswas Ch.** Matrix influence on the tumor cell stimulation of fibroblast collagenase production. J. Cell Biochem. 28: 39-45, 1985.
81 **Liotta LA, Garbisa K, Tryggvason K.** Biochemical mechanisms involved in tumor penetration of the basement membrane. In: Liotta LA, Hart IR (eds.), Tumor invasion and metastasis. Martinus Nijhoff, Boston, pp 319-336, 1984.
82 **Burleigh MC.** Degradation of collagen by non-specific proteinases. In: Barrett (ed.), Proteinases in mammalian cells and tissues Elsevier/North-Holland Biomedical Press pp 285-309, 1977.
83 **Harris ED, Cartwright EC.** Mammalian collagenases. In: Proteinases in mammalian cells and tissues Elsevier (Barrett A J ed.) North-Holland Biomedical Press pp 249-283, 1977.
84 **Weiss JB.** Enzymic degradation of collagen. Int. Rev. Connect. Tissue Res. 7: 101-157, 1976.
85 **Leibovich SJ, and Weiss JB.** Failure of human rheumatoid synovial collagenase to degrade either normal or rheumatoid arthritic polymeric collagen. Biochim. Biophys. Acta 251: 109-188, 1971.
86 **Vater CA, Harris ED jr, Siegel RC.** Native cross-links in collagen fibrils induce resistance to human synovial collagenase Biochem. J. 181: 639-645, 1979.

87 **Scott PG, Goldberg HA, Dodd CM.** A neutral proteinase from human gingival fibroblasts active against the C-terminal cross-linking region of type I collagen. Biochem. Biophys. Res. Commun. 114: 1064-1070, 1983.

88 **Goldberg HA, Scott PG.** Isolation from cultured porcine gingival explants of a neutral proteinase with collagen telopeptidase activity. Connect. Tiss. Res. 15: 209-219, 1986.

89 **Starkey PM, Barrett AJ, Burleigh MC.** The degradation of articular collagen by neutrophil proteinases. Biochim. Biophys. Acta 483: 386-397, 1977.

90 **Starkey PM.** Elastase and cathepsin G, the serine proteinases of human neutrophil leucocytes and spleen. In: Barrett (ed.), Proteinases in mammalian cells and tissues Elsevier North-Holland Biomedical Press pp 57-89, 1977.

91 **Weiss SJ, Peppin GJ.** Collagenolytic metalloenzymes of the human neutrophil. Characteristics, regulation and potential function *in vivo*. Biopolymers 35/19: 3189-3197, 1986.

92 **Birkedal-Hansen H.** Catabolism and turnover of collagens: Collagenases. Methods in Enzymology 144: 140-171, 1987.

93 **Gross J, Harper E, Harris ED jr, McCroskery PA, Highberger JH, Corbett C, Kang AH.** Animal collagenases: Specificity of action and structures of the substrate cleavage site. Biochem. Biophys. Res. Commun. 61: 605-612, 1974.

94 **Gross J, and Lapiere CM.** Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc. Natl. Acad. Sci. USA 48: 1014-1022, 1962.

95 **Blaser J, Knauser V, Osthues A, Reinke H, Tschesche H.** Mercurial activation of human polymorphonuclear leucocyte procollagenase. Eur. J. Biochem. 202/3: 1223-1230, 1991.

96 **Tschesche H, Fedrowitz J, Kohnert U, Michaelis J, Macartney H.** Matrix degrading proteinases from human granulocytes: Type I,II and III collagenase, gelatinase and type IV/V collagenase. A survey of recent findings and inhibition by gamma-anticollagenase. Folia Histochem. Cytobiol. 24/2: 125-132, 1986.

97 **Gross, J.** An assay on biological degradation of collagen. In: Hay (ed.) Cell biology of extracellular matrix. Plenum Press, New York and London pp 217-258, 1981.

98 **Woessner JF jr.** Matrix metalloproteinases and their inhibitors in connective tissue remodelling. FASEB J. 5: 2145-2154, 1991.

99 **Matrisian LM.** The matrix-degrading metalloproteinases. BioEssays 14/7: 455-463, 1992.

100 **Weiss SJ and Peppin GJ.** Collagenolytic metalloenzymes of the

human neutrophil. Characteristics, regulation and potential function *in vivo*. Biochem. Pharmacol. 35/19: 3189-3197, 1986.

101 **Welgus HG, Jeffrey JJ, Eisen AZ.** The collagen substrate specificity of human skin fibroblast collagenase. J. Biol. Chem. 256/18: 9511-9515, 1981.

102 **Sellers A, Reynolds JJ, Meikle MC.** Neutral metalloproteinases of rabbit bone. Separation of latent forms of distinct enzymes that when activated degrade collagen, gelatin and proteoglycans. Biochem. J. 171: 493-496, 1978.

103 **Sopata I, Danciewicz AM.** Presence of a gelatin-specific proteinase and its latent form in human leucocytes. Biochim. Biophys. Acta 370: 510-523, 1974.

104 **Vissers MCM, Winterbourn CC**. Gelatinase contributes to the degradation of glomerular basement membrane collagen by human neutrophils. Collagen Rel. Res. 8: 113-122, 1988.

105 **Murphy G, Reynolds JJ, Bretz U, Baggiolini M.** Partial purification of collagenase and gelatinase from PMN-leucocytes. Analysis of their actions on soluble and insoluble collagens. Biochem. J. 203: 209-221, 1982.

106 **Murphy G, McAlpine CG, Poll CT, Reynolds JJ.** Purification and characterization of a bone metalloproteinase that degrades gelatin and types IV and V collagen. Biochim. Biophys. Acta 831: 49-58, 1985.

107 **Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer J, Kronberger A, He CH, Bauer EA, Goldberg GI.** H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. J. Biol. Chem. 263/14: 6579-6587, 1988.

108 **Murphy G, Ward R, Hembry RM, Reynolds JJ, Kuhn K, Tryggvason K.** Characterization of gelatinase from pig polymorphonuclear leucocytes. A metalloproteinase resembling tumour type IV collagenase. Biochem. J. 258: 463-472, 1989.

109 **Fessler LI, Duncan KG, Fessler JH, Salo T, Tryggvason, K.** Characterization of the procollagen IV cleavage products produced by a specific tumor collagenase. J. Biol. Chem. 259: 9783-9789, 1984.

110 **Murphy G, Hembry RM, McGarrity AM, Reynolds JJ.** Gelatinase (type IV collagenase) immunolocalization in cells and tissues. Use of an antiserum to rabbit bone gelatinase that identifies high and low Mr forms. J. Cell Sci. 92: 487-495, 1989.

111 **Collier IE, Smith J, Kronberger A, Bauer EA, Wilhelm SM, Eisen AZ, Goldberg GI.** The structure of the human skin

fibroblast collagenase gene. J. Biol. Chem. 263/22: 10711-10713, 1988.

112 **Sage H, Woodbury HG, Bornstein P.** Structural studies on human type IV collagen. J. Biol. Chem. 254/19: 9893-9900, 1979.

113 **Liotta LA, Abe S, Gehron Robey P, Martin GR.** Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. Proc. Natl. Acad. Sci. USA 75/5: 2268-2272, 1979.

114 **Murphy G, Cawston TE, Galloway WA, Barnes MJ, Bunning RAD, Mercer E, Reynolds JJ, Burgeson RE.** Metalloproteinases from rabbit bone culture medium degrade types IV and V collagens, laminin and fibronectin. Biochem. J. 209: 741-752, 1983.

115 **Murphy G, Hembry RM, Hughes CE, Fosang AJ, Hardingham TE** Role and regulation of metalloproteinases in connective tissue turnover. Biochem. Soc. Trans. 18: 812-815, 1990.

116 **Galloway WA, Murphy G, Sandy JD, Gravilovic J, Cawston TE, Reynolds JJ.** Purification and characterization of rabbit bone metalloproteinase that degrades proteoglycan and other connective tissue components. Biochem. J. 209: 741-752, 1983.

117 **Murphy G, McGuire MB, Russell RGG, Reynolds JJ.** Characterization of collagenase, other metalloproteinases and an inhibitor (TIMP) produced by human synovium and cartilage in culture. Clin. Sci. 61: 711-716, 1981.

118 **Birkedal-Hansen H.** From tadpole collagenase to a family of matrix metalloproteinases. J. Oral Pathol. 17: 445-451, 1988.

119 **Fini ME, Plucinska IM, Mayer AS, Gross RH, Brinckerhoff CE.** A gene for rabbit synovial cell collagenase: Member of a family of metalloproteinases that degrade the connective tissue matrix. Biochemistry 26/19: 6156-6165, 1987.

120 **Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ.** Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. J. Biol. Chem. 261: 6600-6605, 1986.

121 **Angel P, Baumann I, Stein B, Delius H, Rahmsdorf HJ, Herrlich P.** 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. Mol. Cell. Biol. 7/6: 2256-2266, 1987.

122 **Whitham SE, Murphy G, Angel P, Rahmsdorf HJ, Smith BJ, Lions A, Harris TJR, Reynolds JJ, Herrlich P, Docherty AJP.** Comparison of human stromelysin and collagenase by cloning and sequence analysis. Biochem. J. 240: 913-916, 1986.

123 **Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC, Chambon P.** A novel metallo-

proteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 348: 699-704, 1990.

124 **Matrisian LM, Leroy P, Ruhlman C, Gesnel MC, Breatnach R.** Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. Mol. Cell. Biol. 6: 1679-1686, 1986.

125 **Breatnach R, Matrisian LM, Gesnel MC, Staub A, Leroy P.** Sequences coding for part of oncogene-induced transin are highly conserved in a related rat gene. Nucleic Acid Res. 15/3: 1139-1151, 1987.

126 **Murphy GJP, Murphy G, Reynolds JJ.** The origin of matrix metalloproteinases and their familial relationships. FEBS letters 289(1): 4-7, 1991.

127 **Cambray GJ, Murphy G, Reynolds JJ.** The effects of dexamethasone *in vitro* on the production of collagenase and inhibitor by synovial and cartilage explants from the joints of rabbits with a proliferative arthritis. Rheumatol. Int. 1: 69-72, 1981.

128 **Murphy G, McGuire MB, Russell RGG, Reynolds JJ.** Characterization of collagenase, other metalloproteinases and their inhibitor (TIMP) produced by human synovium and cartilage in culture. Clin. Sci. 61: 711-716, 1981.

129 **Burnett D, Reynolds JJ, Ward RV, Afford SC, Stockley R.** Tissue inhibitor of metalloproteinases and collagenase inhibitory activity in lung secretions from patients with chronic obstructive bronchitis: effect of corticosteroid treatment. Thorax 41: 740-745, 1986.

130 **Marbaix E, Donnez J, Courtroy PJ, Eeckhout Y.** Progesterone regulates the activity of collagenase and related gelatinases A and B in human endometrial explants. Proc. Natl. Acad. Sci. USA 89: 11789-11793, 1992.

131 **Matsusue S, Walser M.** Healing of intestinal anastomoses in adrenalectomized rats given corticosterone. Am. J. Physiol. 263/1: R164-R168, 1992.

132 **Harvey AK, Hrubey PS, Chandrasekhar S.** Transforming growth factor-ß inhibition of interleukin-1 activity involves down-regulation of interleukin-1 receptors on chondrocytes. Exp. Cell Res. 195: 376-385, 1991.

133 **Unemori EN, Hibbs MS, Amento EP.** Constitutive expression of a 92 kD gelatinase (type IV collagenase by rheumatoid synovial fibroblasts and its induction in normal human fibroblasts by inflammatory cytokines. J. Clin. Invest. 88: 1656-1662, 1991.

134 **Martel-Pelletier J, Zafarullah M, Kodama S, Pelletier J-P.** *In vitro* effects of interleukin-1 on the synthesis of

metalloproteinases, TIMP, plasminogen activators and inhibitors in human articular cartilage. J. Rheumatol. Suppl 27 18: 80-84, 1991.

135 **Sasaguri Y, Komiya S, Sugama K, Suzuki K, Inoue A, Morimatsu M, Nagase H.** Production of matrix metalloproteinases 2 and 3 (stromelysin) by stromal cells of giant tumor of bone. Am. J. Pathol. 141: 611-621, 1992.

136 **Takahashi S, Ito A, Nagino M, Mori Y, Xie B, Nagase H.** Cyclic adenosine 3',5'-monophosphate suppresses interleukin-1 induced synthesis of matrix metalloproteinases but not of tissue inhibitor of metalloproteinases in human uterine cervical fibroblasts. J. Biol. Chem. 266/30: 19894-19899, 1991.

137 **Meikle MC, Heath JK, Reynolds JJ.** Advances in understanding cell interactions in tissue resorption. Relevance to the pathogenesis of periodontal diseases and a new hypothesis. J. Oral Pathol 15/5: 239-250, 1986.

138 **Meikle MC, Atkinson SJ, Ward RV, Murphy G, Reynolds JJ.** Gingival fibroblasts degrade type I collagen films when stimulated with tumor necrosis factor and interleukin-1: Evidence that breakdown is mediated by metalloproteinases. J. Periodont. Res. 24: 201-213, 1989.

139 **Shimokado K, Raines EW, Madtes DK, Barrett TB, Benditt EP, Ross, R.** A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell 43: 277-286, 1985.

140 **Circolo A, Welgus HG, Pierce GF, Kramer J, Strunk RC.** Differential regulation of the expression of proteinase/ antiproteinases in fibroblast. J. Biol. Chem. 266/19: 12283-12288, 1991.

141 **Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJP, Angel P, Heath JK.** Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J. 6/7: 1899-1904, 1987.

142 **Girard MT, Matsubaru M, Fini ME.** Transforming growth factor-ß and interleukin-1 modulate metalloproteinase expression by corneal stromal cells. Invest. Ophthalmol Vis Sci 32: 2441-2454, 1991.

143 **Wright JK, Cawston TE, Hazleman BL.** Transforming growth factor beta stimulates the production of the tissue inhibitor of metalloproteinases (TIMP) by human synovial and skin fibroblasts. Biochim. Biophys. Acta. 1094: 207-210, 1991.

144 **Overall CM, Wrana JL, Sodek J.** Transcriptional and post-transcriptional regulation of 72 kDa gelatinase/type IV collagenase by transforming growth factor-ß1 in human

fibroblasts. J. Biol. Chem. 266/21: 14064-14071, 1991.

145 **Heckmann M, Aumailley M, Chu M-L, Timpl R, Krieg T.** Effect of transforming growth factor-ß on collagen type VI expression in human dermal fibroblasts. FEBS 310/1: 79-82, 1992.

146 **Lotz M, Guerne P-A.** Interleukin-6 induces the synthesis of tissue inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). J. Biol. Chem. 266/4: 2017-2020, 1991.

147 **Lacraz S, Nicod L, Galve-de Rochemonteix B, Baumberger C, Dayer J-M, Welgus HG.** Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4. J. Clin. Invest. 90: 382-388, 1992.

148 **Corcoran ML, Stetler-Stevenson WG, Brown PD, Wahl LM.** Interleukin-4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase and 92 Kda type IV collagenase/ gelatinase production by human monocytes. J. Biol. Chem. 267/1: 515-519, 1992.

149 **Richards CD, Rafferty JA, Reynolds JJ, Saklatvala J.** Porcine collagenase from synovial fibroblasts: cDNA sequence and modulation of expression of RNA *in vitro* by various cytokines. Matrix 11: 161-167, 1991.

150 **Mawatari M, Kohno K, Mizoguchi H, Matsuda T, Asoh K-I, van Damme J, Welgus HG, Kuwano M.** Effects of tumor necrosis factor and epidermal growth factor on cell morphology, cell surface receptors, and the production of tissue inhibitor of metalloproteinases and IL-6 in human microvascular endothelial cells. J. Immunol. 143: 1619-1627, 1989.

151 **Brenner DA, O'Hara M, Angel P, Chojker M, Karin M.** Prolonged activation of *jun* and collagenase genes by tumour necrosis factor-α. Nature 337(6208): 661-663, 1989.

152 **Springman EB, Angleton EL, Birkedal-Hansen H, van Wart, HE.** Multiple modes of activation of latent human fibroblast collagenase: Evidence for the role of a Cys^{73} active site zinc complex in latency and a cysteine switch mechanism for activation. Proc Natl Acad Sci USA: 87 364-368, 1990.

153 **Van Wart HE, Birkedal-Hansen H.** The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metallo-proteinase gene family. Proc. Natl. Acad. Sci. USA 87: 5578-5582, 1992.

154 **Crabbe T, Willenbrock F, Eaton D, Hynds P, Carne AF, Murphy G, Docherty AJP.** Biochemical characterization of matrilysin. Activation conforms to the stepwise mechanisms proposed for other matrix metalloproteinases. Biochemistry 31: 8500-8507,

1992.
155 **Nagase H, Ogata Y, Suzuki K, Enghild JJ, Salvesen G.** Substrate specificities and activation mechanisms of matrix metalloproteinases. Biochem. Soc. Trans. 19: 715-718, 1991.
156 **Okada Y, Nakanishi I.** Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 (gelatinase) by human neutrophil elastase and cathepsin G. FEBS letters 249: 353-356, 1989.
157 **Wilhelm OG, Bang NU.** What molecular biology has taught us about fibrinolysis. J. Lab. Clin. Med. 119/2: 111-115, 1992.
158 **Gravilovic J, Murphy G.** The role of plasminogen in cell mediated collagen degradation. Cell Biol. Int. Rep. 13/4: 367-375, 1989.
159 **Reith A, Rucklidge GJ.** Invasion of brain tissue by primary glioma - Evidence for the involvement of Urokinase-type plasminogen activator as an activator of type IV collagenase. Biochem. Biophys. Res. Comm. 186/1: 384-354, 1992.
160 **Schmitt M, Janicke F, Moniwa N, Chucholowski N, Pache L, Graeff H.** Tumor-associated urokinase-type plasminogen activator - Biological and clinical significance. Biol. Chem. Hoppe-Seyler 373/7: 611-622, 1992.
161 **Kobayashi H, Schmitt M, Goreztki L, Chucholowski N, Calevete J, Kramer M, Gunzler WA, Janicke F, Graeff H.** Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (pro-uPA). J. Biol. Chem. 266/8: 5147-5152, 1991.
162 **Goretzki L, Schmitt M, Mann K, Calevete J, Chucholowski N, Kramer M, Gunzler WA, Janicke F, Graeff H.** Effective activation of the proenzyme form of the urokinase type plasminogen activator (pro-uPA) by the cysteine proteinase cathepsin L. FEBS letters 297(1-2): 112-118, 1992.
163 **Murphy G, Cockett MI, Stephens PE, Smith BJ, Docherty AJP.** Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes. Biochem J. 248: 265-268, 1987.
164 **Suzuki K, Enghild JJ, Morodoni T, Salvesen G, Nagase H.** Mechanisms of activation of tissue procollagenase by matrix metalloproteinase-3 (stromelysin). Biochemistry 29: 10261-10270, 1990.
165 **Cawston TE.** Protein inhibitors of metalloproteinases. In: Proteinase inhibitors (Barrett AJ and Salvesen G. eds.) pp 589-610, 1986. Elsevier, Amsterdam.
166 **Kishi J, Hayakawa T.** Purification and characterization of

bovine dental pulp collagenase inhibitor. J. Biochem. 96/2: 395-404, 1984.

167 **Okada Y, Watanabe S, Nakanishi I, Kishi J-I, Hayakawa T, Watorek W, Travis J, Nagase H.** Inactivation of tissue inhibitor of metalloproteinases by neutrophil elastase and other serine proteinases. FEBS letters 229/1: 157-160, 1988.

168 **Stetler-Stevenson WG, Krutzsch HC, Liotta LA.** Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J. Biol. Chem. 264: 17374-17378, 1989.

169 **Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S, He C.** Human 72-kilodalton type IV collagenase forms a complex with tissue inhibitor of metalloproteinases designated TIMP-2. Proc. Natl. Acad. Sci. USA 86: 8207-8211, 1989.

170 **Howard EW, Bullen EC, Banda MJ.** Regulation of the autoactivation of human 72 Kda progelatinase by tissue inhibitor of metalloproteinases-2. J. Biol. Chem. 266: 13064-13069, 1991.

171 **Pavloff N, Staskus PW, Kishnani NS, Hawkes SP.** A new inhibitor of metalloproteinases from chicken: ChIMP-3. J. Biol. Chem. 267/24: 17321-17326, 1992.

CHAPTER 2

COLLAGENOLYTIC ACTIVITY EXTRACTED FROM INTESTINAL ANASTOMOSES OF THE RAT

Jos WJ van der Stappen, Thijs Hendriks and Herman HM de Boer

Matrix 9/1989, pp 238-243

ABSTRACT

The post-operative degradation of collagen has been postulated to play an important role in the development of anastomotic leakage in the intestine. However, collagenolytic activity in intestinal anastomoses has hardly been studied so far. We have measured collagenolytic activity, after extraction in an urea-containing medium, in both ileal and colonic anastomoses in the rat, from 12 hours to 31 days after operation.

In ileum collagenolytic activity increased significantly, from 2 to 4 (average 2.7) times the control value, at 12 hours post-operatively followed by a steady decline to original levels. Four weeks after surgery the activity was still slightly, but significantly, enhanced. In colon collagenolytic activity also increased up to 4 times the pre-operative level (average 3.0) 12 hours after operation. Return to original levels was delayed in colon compared to ileum but here activities were similar to control values after one month. In both parts of the intestine there was only a small increase in activity at a segment proximal to the anastomosis during the first 24 hours after operation. The amount of protein extracted did not vary significantly between control and anastomotic samples. These data are the first to show a transiently increased extractable collagenolytic activity in intestinal anastomoses.

INTRODUCTION

A major complication of surgery of the large bowel is anastomotic leakage. Despite improved surgical techniques healing of colonic anastomoses is disturbed rather frequently (Fielding et al., 1980), resulting in leakage of luminal content into the abdominal cavity and thereby in high mortality and morbidity. The molecular mechanisms underlying this phenomenon still need elucidating in order to find ways of diminishing its frequency.

The tensile strength of the intestinal wall is provided mainly by its connective tissue layer, the submucosa, which

is composed almost entirely of collagen (Gabella, 1987; Thomson et al., 1986). During the first post-operative days anastomotic strength depends solely on the suture holding capacity of the existing collagen fibres in the submucosa. Apart from its function as a structural protein, collagen plays a crucial role in the process of wound healing, e.g. inducing coagulation and acting as a chemoattractant for fibroblasts. It has been shown that collagen concentrations around experimental intestinal anastomoses change massively during the first post-operative days, certainly in the colon (Cronin et al.,1968; Jiborn et al, 1980) and, to a lesser extent, also in the ileum (Hesp et al.,1984; Hendriks et al., 1985). These experimental data are taken as evidence for the existence of a 'collagenous equilibrium' which is crucial to anastomotic healing (Hunt et al., 1980) : after anastomotic construction collagenolysis occurs followed by *de novo* collagen synthesis. If collagen degradation is increased or if synthesis is delayed or its rate too low, restoration of the collagen lattice takes too long and chances for anastomotic leakage increase. Therefore, an understanding of the post-operative collagenolytic process and the enzymes involved, both under normal healing conditions and under circumstances which favour anastomotic dehiscence (Khoury and Waxman, 1983), appears necessary since restriction of collagen degradation might improve intestinal healing. As a first step, we describe the existence of a transient increase in collagenolytic activity, extractable from intestinal anastomoses in the rat.

MATERIALS AND METHODS

Operative Techniques.

Male Wistar rats with an average weight of 220 g (range: 180-280 g) were used. All animals received both an ileal and a colonic anastomosis. Surgery was performed under semi-sterile conditions using a Zeiss operating microscope. Rats were brought under general anaesthesia by an intraperitoneal injection of sodium pentobarbital. The abdomen was opened through a midline incision of

approximately 4 cm. Ileal anastomosis: a small bowel segment 1-2 cm in length, the per-operative control segment, was resected at a distance of 15 ± 3 cm proximal to the ileal-caecal junction (Fig 1:C). An end-to-end anastomosis was constructed using 8 single layer inverting interrupted 8 x 0 Ethilon^{R} sutures (Ethicon, Norderstedt, FRG).

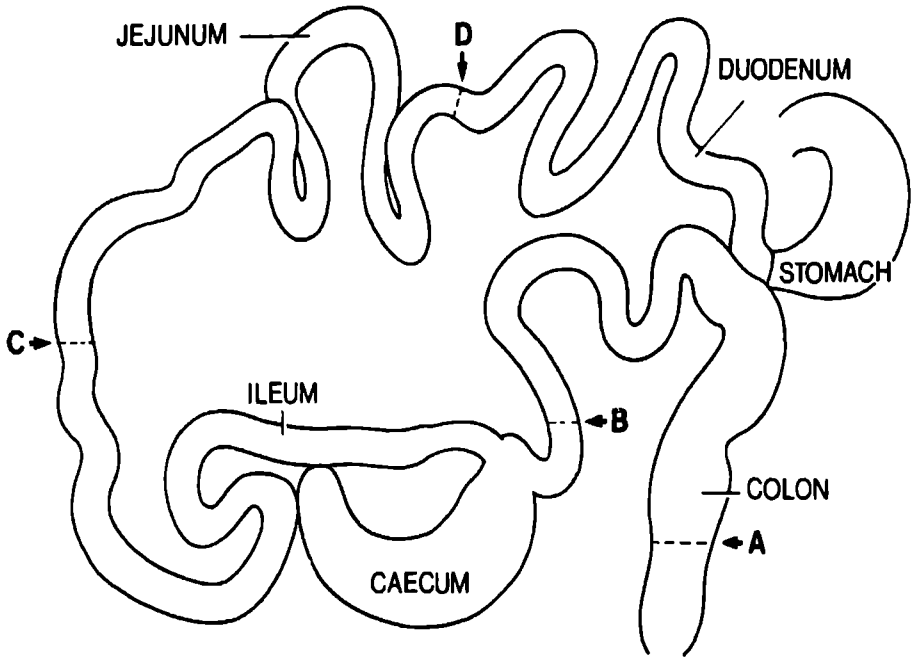

Figure 1: Schematic drawing of the intestinal tract of the rat. Arrows indicate the resection sites. A: Per-operative control segment and anastomosis in large intestine. B: Postoperative control segment in large intestine. C: Peroperative control segment and anastomosis in small intestine. D: Postoperative control segment in small intestine.

Colonic anastomosis : an 1 cm long segment (per-operative control) of the descending colon 3 cm proximal to the peritoneal reflection (Fig 1:A) was removed. Continuity was restored as in ileum. The abdomen was closed in two layers using silk for the fascia and staples for the skin.

After 12 hours (n=6) and 1 (n=7), 2 (n=9), 3 (n=7), 7 (n=11) and 31 (n=6) days the animals were sacrificed by means of an intracardial overdose of sodium pentobarbital and the anastomoses (1 cm) were collected together with post-operative control segments located at approximately 15 cm proximal (Fig 1:D) to the ileal anastomosis and 7 cm proximal (Fig 1:B) to the colonic anastomosis, respectively. Resected tissue segments were opened longitudinally, washed twice in ice-cold physiological salt solution, dried and frozen in liquid nitrogen until further processing.

Preparation of the extract.

Extracts of bowel segments with or without an anastomosis were prepared essentially according to the method of Wirl (1977). Briefly: 100 mg of sample, pulverized in a Braun microdismembrator, was suspended in 2 ml of 10 mM Tris-HCl (pH 7.5), containing 0.4 M sucrose and 5 mM $CaCl_2$ and subsequently rotated for 30 min. Tissue was pelleted by centrifugation at 9000 x g for 15 min and washed twice again. The final sediment was extracted for 2 h with 5 M urea in 50 mM Tris-HCl (pH 7.5), containing 0.2 M NaCl and 5 mM $CaCl_2$. After centrifugation at 40000 x g for 30 min the supernatant was dialysed for 16 h against the same buffer without urea to produce a final urea concentration less than 20 mM. All procedures were performed at 4 °C.

Assay of Collagenolytic Activity.

Collagenolytic activity was assayed by measuring the release of radioactive material from [^{14}C] collagen type I in solution. Pure type I collagen was prepared from pepsin solubilized fetal calf skin, after salt fractionation at neutral pH (Miller and Rhodes, 1982) and labelled with [^{14}C] acetic anhydride according to Cawston and Barrett (1979). Labelled collagen (0.60-1.00 μCi/ml) was dialysed exhaustively against 0.1 M acetic acid in a 50000 D Spectra/Por 6 membrane prior to storage at -30°C.

Assays were carried out in fivefold in 1.5 ml microtubes: 50 μl intestinal extract was added to 20 μl [^{14}C] collagen (2 mg/ml) and 200 μl 50 mM Tris-HCl (pH 7.8), containing 50

mM CaCl2 ,0.5 M NaCl; 50 µl of dialysis buffer (see previous section) was added to control tubes. The reaction was allowed to proceed for 4 h at 37 °C and subsequently terminated by the addition of 50 µl 50 mM Tris-HCl (pH 7.8), containing 0.2 M EDTA. After re-incubation at 40°C for 15 min, to allow denaturation of degradation products, 100 µl of 5 M NaCl/glacial acetic acid (9:1, v/v) was added and caps were placed on ice for at least 30 min (Seng et al.,1984). The tubes were centrifugated at 10000 x g for 10 min and 100 µl of the supernatant was added to 5 ml Aqua Luma (Lumac BV, Landgraaf, The Netherlands) and counted in a liquid scintillation analyzer. Preliminary studies showed enhancement of the collagenolytic activity by addition of APMA, which compound is known to activate latent (pro)collagenase (Stricklin et al.,1983). APMA was therefore routinely included in the assay buffer in a final concentration of 1 mM.

After subtracting the blank, which routinely constituted 4-6% of total counts present, activities were expressed in mU per mg of original wet weight or as mU per mg protein, 1 U representing degradation of 1 µg collagen/min under the conditions stated.

The amount of protein extracted was assayed according to Lowry et al. (1951) using bovine serum albumin as a standard.

Methods used for statistical evaluation of the data are given together with results in the appropriate section.

RESULTS

The urea-based extraction procedure yielded reproducible amounts of solubilized protein from both small and large intestine (Table I). The amounts of protein extracted were similar in colon and ileum. On the whole, variations between animals with respect to protein extracted from per-operative control segments were such, that no significant differences were found between the groups of animals to be sacrificed at the various time points. However, for some unknown reason the yield from the six rats which were to be sacrificed at 31 days, was lower than in the other groups. Little difference was found between per-operative control segments and anastomotic samples. Although mean values of the amount

of protein extracted from control samples were generally higher than those obtained from anastomoses, differences were limited and only significant in a few cases.

Table I: Protein levels in extracts from per-operative control segments and anastomotic segments in the experimental groups. Average values (± S.D.) are expressed as μg/mg tissue wet weight.

		ILEUM		COLON	
days after operation	N	control segment	anastomosis	control segment	anastomosis
0.5	6	48.3 ± 3.5	43.0 ± 8.5	42.6 ± 4.5	38.2 ± 8.5
1	7	48.7 ± 8.1	44.0 ± 7.0	45.4 ± 5.7	40.6 ± 4.9[1]
2	9	47.4 ± 7.1	42.9 ± 4.7[1]	42.6 ± 7.7	44.5 ± 7.2
3	7	50.6 ± 5.4	48.2 ± 6.4	49.2 ± 7.5	47.6 ± 5.5
7	11	50.8 ± 5.0	44.4 ± 6.1[1]	48.1 ± 3.6	48.1 ± 5.4
31	6	37.4 ± 1.3	36.5 ± 7.7	39.2 ± 2.5	38.9 ± 3.9
total	46	47.1 ± 6.4	43.2 ± 7.3	44.6 ± 6.8	42.7 ± 7.4

[1] Significant ($0.01 < p < 0.05$; Wilcoxon signed rank test) difference between anastomosis and control segment.

Table II: Collagenolytic activity in extracts from previously uninjured intestine. Data are given as mean value ± S.D.

		ILEUM (per-operative segment)		COLON (per-operative segment)	
Days	N	mU/mg protein extracted	mU/mg wet weight extracted	mU/mg protein extracted	mU/mg wet weight extracted
0.5	6	53.4 ± 22.7	2.6 ± 1.0	44.8 ± 24.2	1.9 ± 1.2
1	7	63.9 ± 29.1	3.0 ± 1.5	53.1 ± 29.1	2.4 ± 1.3
2	9	45.3 ± 27.2	2.1 ± 1.3	35.2 ± 20.1	1.4 ± 0.8
3	7	58.4 ± 20.4	2.8 ± 0.8	39.2 ± 12.2	1.9 ± 0.3
7	11	47.4 ± 18.4	2.3 ± 0.9	41.6 ± 21.6	2.0 ± 1.1
31	6	44.5 ± 27.3	1.7 ± 1.0	25.9 ± 10.8	1.0 ± 0.4
total	46	51.6 ± 23.8	2.4 ± 1.1	39.3 ± 22.5	1.7 ± 1.0

Collagenolytic activity in the urea extracts from bowel biopsies was expressed as mU per mg wet weight used for extraction or as mU per mg of protein extracted. Similar results were found in both cases. Table II shows the activities extracted from the control tissue segments resected at operation, presumably representing uninjured intestine. All assays were performed in the presence of

APMA. If APMA was omitted from the incubation there was hardly any activity detectable in colonic samples. Addition of APMA resulted in measurable activities in almost all intestinal samples: in 4 out of 46 cases collagenolytic activity was not detectable in per-operative colon control samples but was measurable in the anastomotic and post-operative segments. The effect of APMA stimulation on ileal extracts was less pronounced, indicating an increased amount of active enzyme in these extracts. Here, measurable activities were found in all extracts also if APMA was omitted. Other extraction procedures (Curry et al.,1986; Beckhout et al.,1986) were also performed but yielded less activity.

Basal activities appear to be somewhat higher in ileum than in colon. Clearly, a large inter-animal variation occurs. Thus, it is important to compare post- and per-operative activities within one animal, each rat serving as its own control.

Figures 2 and 3 depict, for each rat, the ratio between anastomosis and per-operative control (A) and the ratio between post-operative control and per-operative control (B) in ileum and colon, respectively. The values shown were calculated on the basis of activities expressed as mU/mg protein extracted. Figures where data were calculated from activities per mg wet weight used for extraction are very similar and consequently not shown. Clearly, more collagenolytic activity could be extracted from anastomotic tissue than from uninjured intestine. The average increase at each post-operative time point is given in table III. In ileum, collagenolytic activity was elevated significantly from 12 hours to 31 days after operation. The increase was most pronounced in the first post-operative period. A similar trend was found in colon. Here, activities extracted from 31 days-old anastomoses were no longer higher than those extracted from the corresponding per-operative control segments. Collagenolytic activities in the post-operative control segment from ileum were at no time point significantly different from those extracted from the per-operative control segment (Fig 2B). In colon, collagenolytic activity in the post-operative control segment was significantly elevated at 12 and 24 hours after

operation (Fig 3B, p= 0.031, Wilcoxon signed rank test).

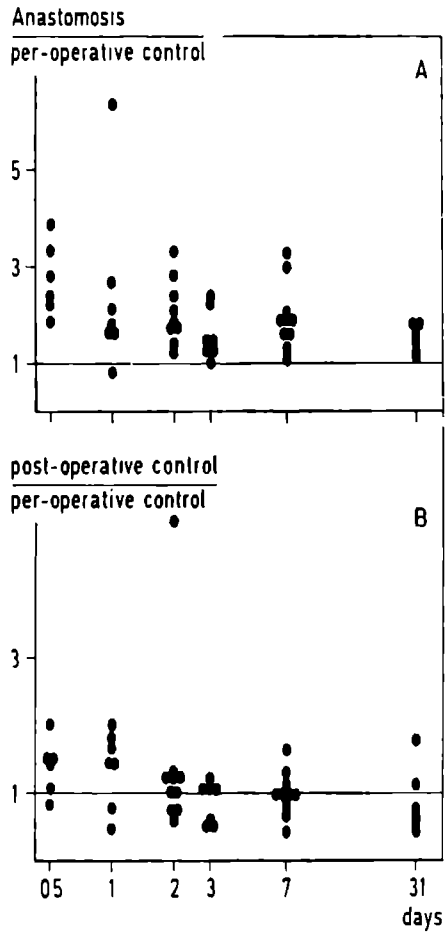

Figure 2:

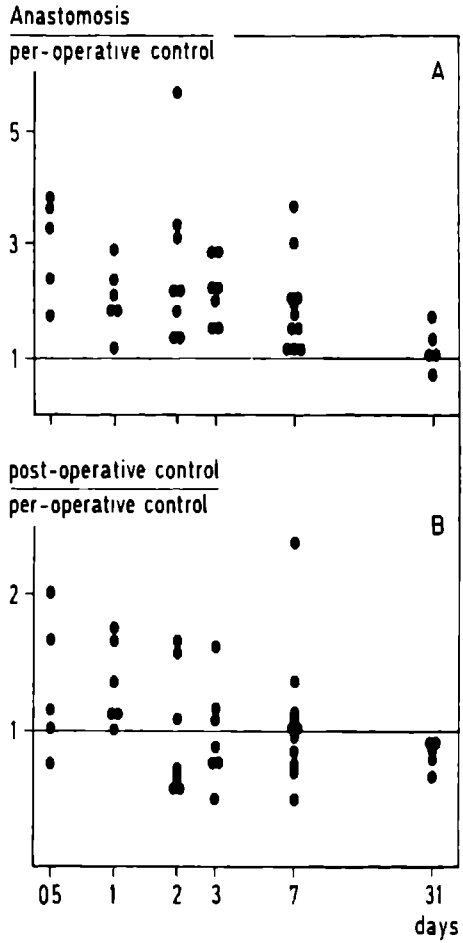

Figure 3:

Figure 2: Relative changes in collagenolytic activity in **ileum** during the first month after operation. Values are expressed as the ratio between activity extracted from the anastomosis and the activity extracted from the peroperative control segment (A) and the ratio between activities extracted from the post-operative and the peroperative control segment, respectively (B). Separate points represent measurements within one rat.

Figure 3: Relative changes in collagenolytic activity in **colon** during the first month after operation. See legend Figure 2.

Table III: Increased collagenolytic activity in anastomotic extracts. The average ratio of activity extracted from the anastomosis in relation to activity extracted from the per-operative control segment is given (± S.D.) Ratios are based on enzyme activities in mU/mg protein.

Days after operation	N	ILEUM	p-value	COLON	p-value
0.5	6	2.73 ± 0.74	0.031	2.95 ± 0.89	0.031
1	7	2.43 ± 1.83	0.031	2.01 ± 0.58	0.016
2	9	2.07 ± 0.67	0.004	2.58 ± 1.43	0.004
3	7	1.59 ± 0.51	0.016	2.13 ± 0.54	0.016
7	11	1.90 ± 0.68	0.001	1.88 ± 0.79	0.012
31	6	1.53 ± 0.29	0.031	1.15 ± 0.37	0.220

Level of significance was calculated using Wilcoxon's signed rank test.

DISCUSSION

It is generally accepted that repair of the collagenous interstitial network is an essential feature of the wound healing sequence (Forrest, 1983). Since tissue collagen levels are a net result of the separate processes of collagen degradation and collagen synthesis, the balance between the two processes is of importance to the healing wound. Collagen degradation after injury has been investigated much less than collagen biosynthesis. Attempts to improve intestinal healing by influencing anastomotic collagen metabolism should be preceded by characterization of the separate processes of collagen degradation and synthesis in this particular tissue.

Only a few reports exist concerning collagenolytic activity in uninjured intestine (Oyamada et al.,1983 ;Lewin et al.,1986; Irimura et al.,1987). Since we encountered great difficulties in preventing massive growth of bacteria in explants from rat intestine, we limited our present efforts to the measurement of activity in extracts from intestinal tissue. Table II shows that collagenolytic activity was indeed present in urea-extracts from both ileum and colon. Although the activities measured are small, and the labelled collagen blank in the assays may comprise up to 50% of total counts released, the sensitivity of the method is sufficient to detect even relatively small changes. It should be emphasized that all assays were run

in the presence of APMA and thus represent the sum of latent and active enzyme activities. While under our assay conditions degradation of type I collagen is probably not mediated exclusively by true collagenases (Birkedal-Hansen ,1987), the fact that activities are enhanced by the presence of APMA and inhibited by both EDTA and 1,10-O-phenanthroline [van der Stappen: unpublished results] indicates the involvement of a metalloproteinase.

The occurrence of collagenolysis during the healing of intestinal anastomoses has been taken for granted (Hunt et al.,1980; Khoury and Waxman,1983). Still, with the exception of the work of Hawley et al. (1970), who reported increased collagenolytic activity in colonic anastomoses 3 days after operation as measured by a semi-quantative lysis assay, no experimental data concerning possible mechanisms of this process had been available. Our results prove that extractable collagenolytic activity is significantly, and transiently, increased after operation. Very recently, Chowcat et al. (1988), in an immunohistochemical study using monoclonal antibodies, showed the presence of extracellular collagenase in rabbit colonic anastomoses. The essence of their results corresponds quite well with our data, despite the obvious difference in methodology, the species difference and the fact that they constructed everted anastomoses, which are hardly ever used in intestinal surgery.

The activities measured do not necessarily reflect the actual increase in collagenolytic potential in anastomotic tissue. It seems quite possible that the injury-induced increase in collagenolytic activity we measured is underestimated because a substantial part of the enzyme molecules could have been bound by TIMP and thus escape detection (Reynolds, 1985). Since immunohistochemistry indicates the precise location of antigens but does not allow conclusions regarding their actual activity, we feel that our data are complementary to those reported by Chowcat et al. (1988) and not merely confirm their very recent findings. Moreover, our study has shown that changes in collagenolytic activity also, and to the same extent, occur in ileal anastomoses where the clinical frequency of leakage is far below that reported for the large bowel.

The present results, which show an increased capacity for collagen degradation during the first week after construction of intestinal anastomoses in the rat, support the hypothesis that the decreased collagen concentrations measured before around colonic (Jiborn et al.,1980) and ileal (De Roy van Zuidewijn et al.,1986) anastomoses in this animal, indeed reflect a process of collagen breakdown. It should be emphasized that our system, based on established surgical procedures, is one of the few existing which enables the study of tissue degrading processes under non-pathological conditions. It remains to be established which enzymes are involved. We are currently applying lower temperatures, fibrillar substrates and specific inhibitors in the assay of anastomotic extracts in order to confirm the presumed participation of an active collagenase. Thereafter, this system will be used to study the postulated increase of collagenolytic capacity in anastomoses, constructed under conditions which increase chances for anastomotic dehiscence. Such research appears essential in order to find measures to limit anastomotic leakage.

Footnotes : EDTA : Ethylenediaminetetraacetic acid ; APMA : p-Aminophenyl-mercuric acetate ; TIMP : Tissue Inhibitor of Metalloproteinases.

LITERATURE.

Birkedal-Hansen, H.: Catabolism and turnover of collagens: collagenases. Methods in Enzymology 144, 140-171, 1987.
Cawston, T.E. and Barrett ,A.J.: A rapid reproducible assay for collagenase using 14C acetylated collagen. Anal. Biochem. 99: 340-345, 1979.
Chowcat,N.L., Savage,F.J., Hembry,R.M. and Boulos,P.B.: Role of collagenase in colonic anastomoses: a reappraisal. Br. J. Surg. 5: 330-334, 1988.
Cronin, K., Jackson, D.S. and Dunphy, J.E.: Changing bursting strenght and collagen content of the healing colon. Surg. Gynecol. Obstet. 126: 747-753, 1968.
Curry, T.E., Clark, M.R., Dean, D.D., Woessner, J.F. and LeMaire, W.J.: The preovulatory increase in ovarian collagenase activity in the rat is dependent of prostaglandin production.Endocrinology 118: 1823-1828, 1986.
Eeckhout, Y., Delaissé, J.-M. and Vaes, G.: Direct extraction and assay of bone tissue collagenase and its relation to parathyroïd -hormone-induced bone resorption. Biochem. J. 239: 793-796, 1986.
Fielding, L.P., Stewart-Brown, S., Blesovsky, L. and Kearney, G. Anastomotic integrity after operations for large-bowel cancer: a multicentre study. Br. Med. J. :411-414, 1980.

Forrest, L.: Current concepts in soft connective tissue wound healing. Br. J. Surg. 70: 133-140, 1983.
Gabella, G.: The cross-ply arrangement of collagen fibres in the submucosa of the mammalian small intestine. Cell Tissue Res. 248: 491-497, 1987.
Hawley, P.R., Faulk, W.P., Hunt, T.K. and Dunphy, J.E.: Collagenase activity in the gastro-intestinal tract. Br. J. Surg.57: 896-904, 1970.
Hendriks, Th., Vereecken, T.H.L.B., Hesp, W.E.L.M., Schillings, P.H.M. and Boer de, H.H.M.: Loss of collagen from experimental intestinal anastomoses : early events. Exp. Mol. Pathol. 42: 411-418, 1985.
Hesp, W.L.E.M., Hendriks, Th., Lubbers, E.J.C. and Boer de, H.H.M.: Wound healing in the intestinal wall. A comparison between experimental ileal and colonic anastomoses. Dis. Colon Rectum 27:99-104, 1984.
Hunt,T.K., Hawley,P.R., Male,J., Goodson,W., Thakral,K.K. : Wound healing and wound infection, ed. by Hunt,T.K., Appleton-Century-Crofts, New York, 1980, pp 153-159.
Irimura, T., Yamori, T., Bennett, S.C., Ota, D.M. and Cleary, K.R.: The relationship of collagenolytic activity to stage of human colorectal carcinoma. Int. J. Cancer 40: 24-31, 1987.
Jiborn, H., Ahonen, J., Zederfeldt, B.: Healing of experimental colonic anastomosis III collagen metabolism in the colon after left colon resection. Am. J. Surg. 139: 398-405, 1980.
Khoury, G.A. and Waxman, B.P.: Large bowel anastomoses. I. The healing process and sutured anastomoses- a review. Br. J. Surg. 70, 61-63,1983.
Lewin, M.R., Chowcat, N.L., Jayaraj, A.P. and Boulos, P.B.: Collagenase inhibition in colonic mucosa by proteinase inhibitors. Br. J. Exp. Path. 67: 523-526,1986.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275, 1983.
Miller, E.J. and Rhodes, R.K.: Preparation and characterization of the different types of collagen. Methods in Enzymology 82A: 33-64, 1982.
Oyamada, I., Kitaoka, M., Araki, C., Yoshioka, H. and Usuku, C.: Collagenase from the culture medium of rabbit-colon wall, with special reference to the latent type and substrate specificity. Gastroenterology 85: 376-384,1983.
Reynolds, J.J.: The molecular and cellular interactions involved in connective tissue destruction. Br. J. Dermatol. 112: 715-723, 1985.
de Roy van Zuidewijn, D.B.W., Wobbes, Th., Hendriks, Th., Klompmakers, A.A. and Boer de,H.H.M.: The effect of antineoplastic agents on the healing of small intestinal anastomoses in the rat. Cancer 58: 62-66, 1986.
Seng, W.L., Davison, P.F. and Kenyon, K.R.: A simple vertebrate collagenase assay using soluble radioactive collagen substrate. Anal. Biochem. 139: 17-24, 1984.
Stricklin, G.P., Jeffrey, J.J., Roswit, W.T. and Eisen, A.Z.: Human skin fibroblast pro collagenase: mechanism of activation by organomercurials and trypsin. Biochemistry 22: 61-68, 1983.
Thomson, H.J., Busuttil, A., Eastwood, M.A., Smith, A.N. and Elton, R.A.:The submucosa of the human colon.J. Ultrastruc. Mol.

Struc. Res. 96: 22-30, 1986.
Wirl, G.: Extractable collagenase and carcinogenesis of mouse skin. Connect. Tiss. Res. 5: 171-178, 1977.

CHAPTER 3

COLLAGENOLYTIC ACTIVITY IN EXPERIMENTAL INTESTINAL ANASTOMOSES

Differences between small and large bowel and evidence for the presence of collagenase.

Jos W.J. van der Stappen, Thijs Hendriks, Herman H.M. de Boer, Ben M. de Man and Jan Joep H.H.M. de Pont

Int. J. Colorect. Dis. 7: pp 95-101, 1992

ABSTRACT

Collagen degradation is thought to be an integral part of the healing sequence of intestinal anastomoses, but almost nothing is known about the enzyme activities involved. We have studied collagenolytic activities, extracted from 1 day-old intestinal anastomoses in the rat. Using either soluble type I collagen or fibrillar type I or type III collagen as a substrate, activities measured in extracts from anastomotic segments were compared to those in extracts from uninjured intestine, removed at operation: in all cases, the collagenolytic activity in anastomotic extracts was higher. This increase was significantly more pronounced in large bowel than in small bowel. The activities were strongly inhibited by serum and metallo-chelating compounds. Analysis, by means of SDS-polyacryl-amide gel electrophoresis, of the reaction products of the degradation of fibrillar type I collagen by the extracts revealed the presence of a multitude of fragments, amongst them T_cA fragments characteristic for the activity of mammalian collagenase. Thus, the degradative capacity towards various collagen substrates is enhanced in the anastomotic area during the first postoperative period and a true mammalian collagenase is one of the enzymes present.

INTRODUCTION

Anastomotic leakage remains one of the major complications of gastro-intestinal surgery. This phenomenon occurs relatively frequently in the large bowel [1], while leakage of small bowel anastomoses is rarely observed [2]. Clinically manifest leakage rates for colonic anastomoses have been reported to be as high as 14% [3]; inspection by X-rays of extraperitoneal anastomoses even revealed leakage rates of up to 51% [4].

The necessity of including the submucosa, as the major structural layer of the intestinal wall, in the suturing of the bowel ends was recognized a century ago [5]. This layer is made up almost entirely of collagen [6,7] and therefore collagen metabolism is crucial to anastomotic strength [8]. After anastomotic construction, collagen degradation is

thought to take place to some extent, as a consequence of the inflammatory reaction which is an essential part of the normal wound healing sequence. Infiltrating neutrophils, characteristic for early inflammation, could be involved in this process. They comprise not only a potent source of collagen degrading enzymes, such as collagenase and gelatinase, but are also known as an important source of collagenase activators [9].

Since sutures have to be anchored primarily in the submucosal collagenous network, degradation of collagen fibrils may weaken anastomotic strength, sometimes resulting in anastomotic dehiscence. Under certain conditions, e.g. the presence of infection, collagen degradation may become more extensive leading to diminished strength and enhanced risk of anastomotic rupture [10]. Therefore, an understanding of the degradative processes involved appears necessary to find ways to prevent this type of complication in intestinal surgery.

Very little is known about the process of collagen degradation around intestinal anastomoses. Its occurrence may be derived from post-operative changes in collagen concentration and content [11-13] but so far its mechanism has hardly been investigated. Early experiments by Hawley et al. [14] suggested the participation of collagenolytic enzymes and, very recently, the presence of collagenase activity was indeed demonstrated immunohistochemically in colonic anastomoses of the rabbit [15]. In a previous study we have reported the presence of collagenolytic activity in extracts from rat intestinal anastomoses [16]. This activity, which is transiently increased after operation, was measured using soluble collagen as a substrate, leaving the possibility that it originates from non-specific proteases. In the present paper, we further characterize this collagenolytic activity by using fibrillar collagen substrates.

MATERIAL AND METHODS

Operation

Male Wistar rats with an average weight of 200 ± 25 gram

received both an ileal and a colonic anastomosis after resection of a 1 cm long segment (peroperative control). Surgical procedures were as previously described [16]. End-to-end anastomoses were constructed microsurgically, using single-layer inverting interrupted sutures (8 x 0 monofilament polyamide, Ethilon^{R}), approximately 15 cm proximal to the ileal-caecal junction and 3 cm proximal to the peritoneal reflection, respectively. After 24 h the animals were killed by means of an overdose of sodium pentobarbital and the anastomotic segments (approximately 1 cm) were collected. Resected segments were opened longitudinally, washed thoroughly with ice-cold physiological salt solution, dried and frozen in liquid nitrogen until further processing.

Extraction

Extracts of bowel segments with or without an anastomosis were prepared essentially according to Wirl [17]. Briefly: 100 mg of sample, pulverized in a Braun microdismembrator, was suspended in 2 ml of 10 mM Tris-HCl (pH 7.5) containing 0.4 M sucrose and 5 mM $CaCl_2$ and subsequently rotated for 30 min. Tissue was sedimented by centrifugation at 9000 x g for 15 min and washed twice again. The final sediment was extracted for 2 h with 5 M urea in 50 mM Tris-HCl (pH 7.5), containing 0.2 M NaCl and 5 mM $CaCl_2$. After centrifugation at 40000 x g for 30 min the supernatant was dialysed for 16 h against the same buffer without urea. All procedures were performed at 4°C.

Analytical procedures

Collagenolytic activity was measured both in a soluble assay using ^{14}C-labelled type I collagen and in two assays using ^{14}C-labelled reconstituted type I and type III fibrils, respectively, as a substrate. Collagenolytic activity was determined by measuring the release of radioactive material from the collagen substrates. Type I collagen used in the soluble assay and type III collagen used in the fibrillar assay were purified from pepsin-solubilized fetal calf skin. Type I collagen used in

the fibrillar assay was purified from the acid-soluble fraction of fetal calf skin. The collagens were purified by differential salt fractionation at neutral pH [18] and labelled with [^{14}C]-acetic anhydride (25 mCi/mmol, Amersham, UK) [19].

Labelled collagens were dialysed exhaustively against 0.1 M acetic acid in a 50000 D Spectra/Por 6 membrane prior to storage at -30°C. The soluble assays was performed as previously described [16]. For fibrillar assays, collagen type I or III solutions in acetic acid were neutralized by dialysis against 30 mM Tris-HCl (pH 7.3), containing 30 mM $Na_2HPO_4.2H_2O$ and 135 mM NaCl. Aliquots of 20 μl neutralized collagen solution were applied to each well of a 96 wells plastic tissue culture plate (Greiner High Affinity plates; Greiner, Alphen a/d Rijn, The Netherlands) and allowed to gel for 1 h in a humidified atmosphere at 37°C [20]. After gelling the plates were air-dried in a laminar flow hood. Dried plates were washed twice with demineralized water to remove remnants of buffer.

Assays were carried out in triplicate: 50 μl of the extracts and 150 μl of 50 mM Tris-HCl (pH 7.8), containing 50 mM $CaCl_2$ and 0.5 M NaCl were applied to each well. The reaction was allowed to proceed for 22 h at 37°C in a humidified atmosphere to avoid evaporation. At the end of the incubation period the whole content of each well was transferred into microtubes and centrifuged at 10000 x g for 10 min. A 100 μl sample of the supernatant was mixed with 3 ml of Aqua Luma (Lumac BV, Landgraaf, The Netherlands) and counted in a liquid scintillation analyzer. The organomercurial p-aminophenylmercuric acetate (APMA) was routinely added to the incubations in a final concentration of 1 mM in order to activate latent collagenase. In some experiments, protease inhibitors were added to the assay buffer for characterization purposes: Soybean trypsin inhibitor was purchased from Sigma, St. Louis, USA; Phehylmethylsulphonylfluoride (PMSF) was obtained from Serva, Heidelberg, FRG; Trasylol (aprotinin) was obtained from Bayer, Leverkusen, FRG; Tissue inhibitor of metalloproteinases TIMP was a gift from Dr. G. Murphy from Strangeways Research Laboratory, Cambridge, UK). Collagenolytic activities were expressed as mU per mg of

original wet weight or as mU per mg protein extracted, 1 U representing the degradation of 1 μg collagen/min under the conditions stated.

For sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), extraction volumes (4 ml/100 mg of pulverized tissue) obtained from anastomoses from 4 rats were pooled and concentrated to approximately 500 μl. Fibrillar type I assay supernatants from 6 wells, obtained after incubation of 75 μl concentrated extract with 225 μl assay buffer and 20 μl [^{14}C] labelled collagen for 72 h at 28°C, were collected and pooled. The pools were dialysed overnight against demineralized water and lyophilized. Lyophilized material was separated by SDS-PAGE using 10 or 8% polyacrylamide [21]. Protein bands were visualized by standard autoradiography.

The amount of protein extracted was assayed according to Lowry et al. [22] using bovine serum albumin as a standard. Statistical methods are mentioned with the results.

RESULTS

The collagenolytic activity in the urea extracts from bowel biopsies was calculated on the basis of both mg wet weight used for extraction (Table 1) and mg protein extracted (Table 2). Both approaches revealed similar results. The existence of large inter-animal variations, apparent from the relatively high standard deviations, emphasizes the necessity of comparing anastomotic and control values within the same animal. Basal activities in uninjured intestine, i.e. activities extractable from the control segments, were higher in ileum than in colon. Statistical evaluation proved this to be the case in all three assays employed.

Figure 1 shows the post-operative change in collagenolytic activity for each rat, calculated as the ratio between activities measured in anastomotic and control segments. Despite the fact that each animal acted as its own control, great variations were found between animals. Collagenolytic activity extracted from colonic anastomoses was, in almost all rats, higher than the activity measured in extracts from the control segments. This effect was less

Table 1 : Collagenolytic activity, expressed on the basis of wet weight, extractable from control and anastomotic segments. Average values (± S.D) are expressed as mU/mg wet weight.

	SUBSTRATES		
	Soluble type I	**Fibril type I**	**Fibril type III**
Ileum			
Control	2.30 ± 0.70 (n= 27)	2.04 ± 0.90 (n= 24)	1.58 ± 0.83 (n= 23)
Anastomoses	2.68 ± 0.73 (n= 27)	2.54 ± 1.17 (n= 24)	1.67 ± 0.80 (n= 24)
Colon			
Control	1.86 ± 0.77 (n= 27)	1.24 ± 0.73 (n= 22)	1.08 ± 0.62 (n= 23)
Anastomoses	2.46 ± 0.62 (n= 26)	1.80 ± 0.80 (n= 23)	1.35 ± 0.70 (n= 23)
p-value*	0.0010	0.0001	0.0001

* The p-value of the difference between ileal and colonic control samples was calculated using Wilcoxon's signed rank test.

Table 2 : Collagenolytic activity, expressed on the basis of protein, extractable from control and anastomotic segments. Average values (± S.D) are expressed as mU/mg protein extracted.

	SUBSTRATES		
	Soluble type I	**Fibril type I**	**Fibril type III**
Ileum			
Control	68.2 ± 20.5 (n= 27)	60.2 ± 27.3 (n= 24)	45.2 ± 2.8 (n= 24)
Anastomoses	81.3 ± 24.4 (n= 27)	73.2 ± 28.4 (n= 24)	47.9 ± 21.1 (n= 24)
Colon			
Control	58.2 ± 23.1 (n= 27)	38.3 ± 22.1 (n= 22)	32.2 ± 16.3 (n= 24)
Anastomoses	90.3 ± 26.8 (n= 26)	62.5 ± 25.1 (n= 23)	44.9 ± 20.6 (n= 24)
p-value*	0.0237	0.0002	0.0003

* The p-value of the difference between ileal and colonic control samples was calculated using Wilcoxon's signed rank test.

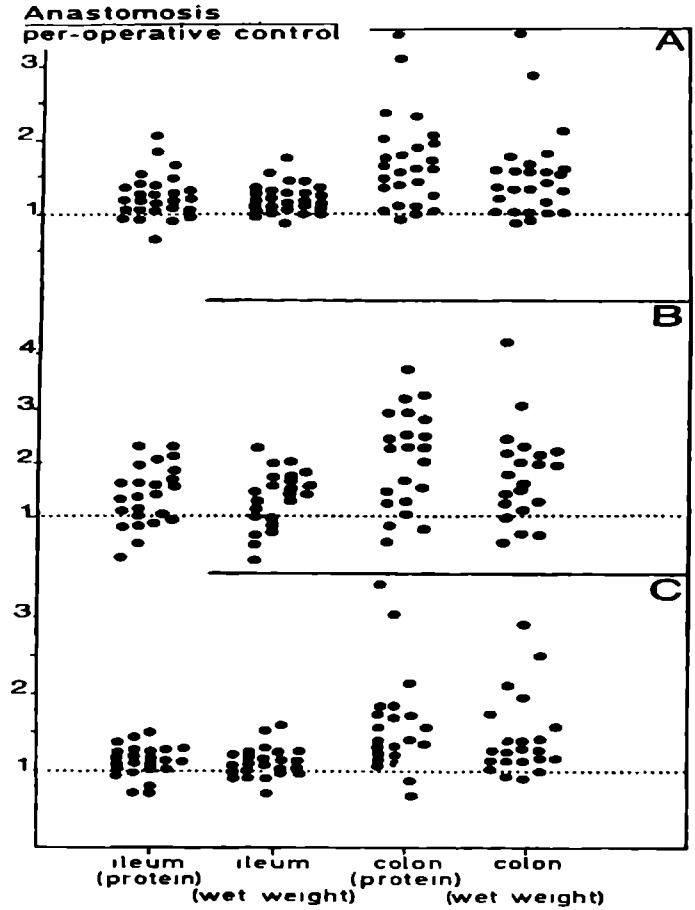

Figure 1. Relative change in collagenolytic activity extractable from 1 day-old intestinal anastomoses. Collagenolytic activity was measured using soluble collagen type I (A), fibrillar type I (B) and fibrillar type III (C) as a substrate. Values are expressed as the ratio between the activity extracted from the anastomosis and the activity extracted from the per-operative control segment. Separate points represent measurements within one rat.

pronounced in ileum. The increased activity in anastomotic extracts , measured before using a soluble substrate, was also found when fibrillar substrates were employed. The average increase in anastomotic collagenolytic activity was calculated, both if activities were expressed on a wet weight basis (Table 3) and if activities were expressed on a protein basis (Table 4).

Table 3: Post-operative increase in collagenolytic activity measured if activities are expressed on the basis of wet weight. The ratio between activities extracted from anastomotic and control segments, calculated for each rat, is given as the average ± S.D.

	SUBSTRATES		
	Soluble type I	**Fibril type I**	**Fibril type III**
Ileum Ratio	1.19 ± 0.09 (n= 27)	1.32 ± 0.51 (n= 24)	1.10 ± 0.19 (n= 24)
p-value*	0.0001	0.0068	0.0116
Colon Ratio	1.61 ± 1.11 (n= 26)	1.76 ± 0.85 (n= 21)	1.43 ± 0.51 (n= 22)
p-value	0.0001	0.0010	0.0002
Difference ileum/colon			
p-value*	0.0084	0.0762	0.0082

* p-values were calculated using Wilcoxon's signed rank test.

Table 4: Post-operative increase in collagenolytic activity measured if activities are expressed on a protein basis. The ratio between activities extracted from anastomotic and control segments, calculated for each rat, is given as the average ± S.D.

	SUBSTRATES		
	Soluble type I	**Fibril type I**	**Fibril type III**
Ileum Ratio	1.22 ± 0.30 (n= 27)	1.31 ± 0.52 (n= 24)	1.11 ± 0.20 (n= 24)
p-value*	0.0059	0.0140	0.0158
Colon Ratio	1.73 ± 0.82 (n= 26)	2.01 ± 0.88 (n= 21)	1.62 ± 0.81 (n= 22)
p-value*	0.0001	0.0004	0.0002
Difference ileum/colon			
p-value*	0.0006	0.0239	0.0061

* p-values were calculated using Wilcoxon's signed rank test.

Statistical analysis of these data revealed a significant increase in collagenolytic activity measured in the extracts of 1-day-old anastomotic segments compared to that measured in extracts from the peroperative controls. This effect was observed with all the three substrates and independent of the manner in which activity was expressed. In addition, the

increase was significantly more pronounced in colonic than in ileal anastomoses.

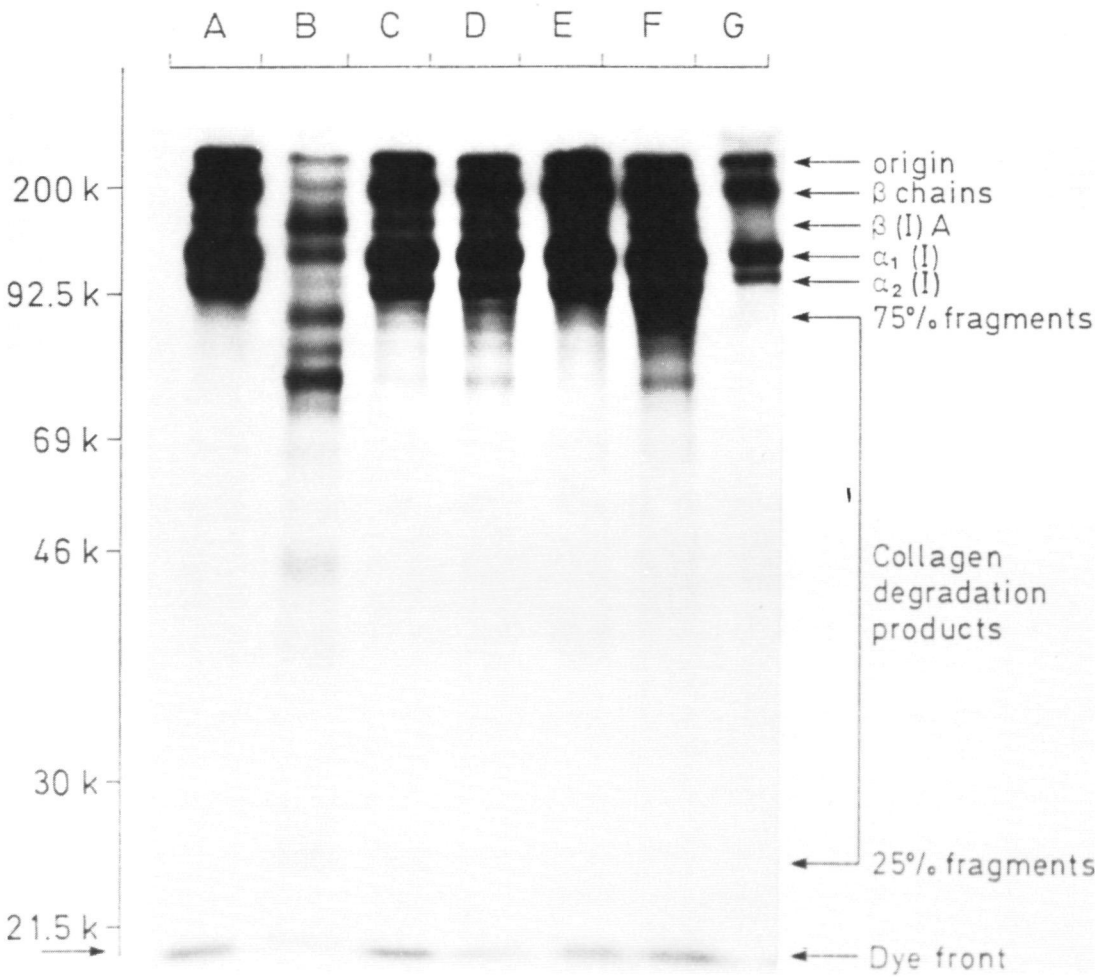

Figure 2. SDS-Polyacrylamide gel electrophoresis of the lysis products obtained from the incubation (72 h at 28°C) of ^{14}C-labelled fibrillar type I collagen with anastomotic extracts. Solubilized material was lyophilized and applied to a 10% polyacrylamide gel; protein bands were visualized by autoradiography. Lanes A,C and E: lysis products produced by colon anastomotic extracts from three different rats. Lanes B,D and F: lysis products produced by the corresponding ileal anastomotic extracts. Lane G : control type I collagen. Molecular weight values are given to the left of the gel.

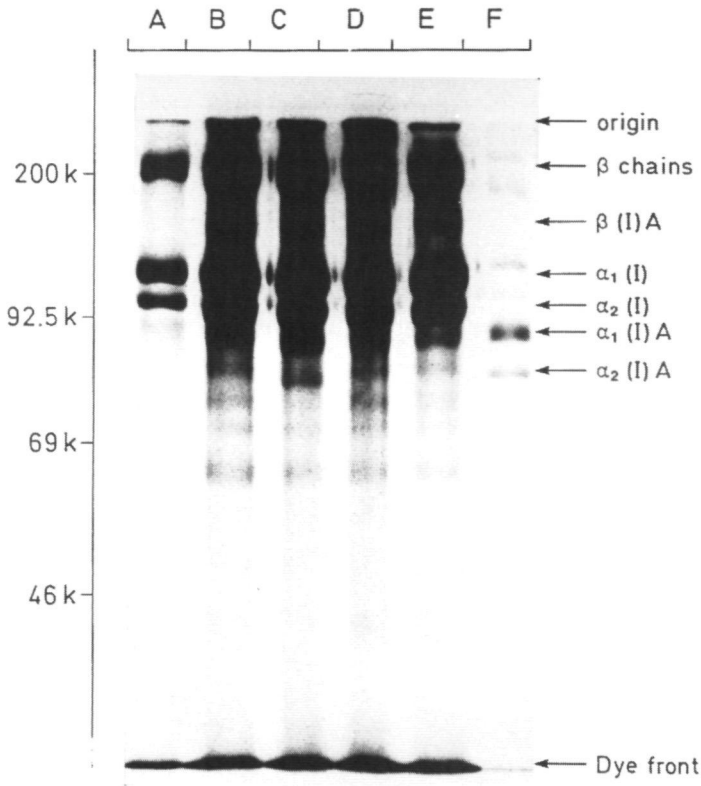

Figure 3: SDS-Polyacrylamide gel electrophoresis of the lysis products obtained from the incubation (72 h at 28°C) of ^{14}C-labelled fibrillar type I collagen with rat anastomotic extracts. Solubilized material was lyophilized and applied to a 8% polyacrylamide gel; protein bands were visualized by autoradiography. Lane A: type I collagen; lanes B and D: lysis products produced by ileal anastomotic extracts from two different rats; lanes C and E: lysis products produced by the corresponding colonic anastomotic extracts; lane F: lysis products obtained from an incubation (22 h at 28°C) of collagen type I fibrils with purified human fibroblast collagenase, showing the characteristic T_cA α_1(I)A and α_2(I)A fragments respectively. Molecular weight values are given to the left of the gel.

In order to characterize the collagenolytic activity in the intestinal extracts we have separated the reaction products of the degradation of fibrillar type I collagen by means of SDS-polyacrylamide gel electrophoresis. In Fig. 2 the intact substrate and its lower molecular weight lysis products are visualized by autoradiography. Lysis products

can be seen between the intact dimers (ß-chains) and monomers (α-chains) and between the monomers and the dye front. The smallest lysis products which were retarded by the 10% polyacrylamide gel ran together with the tracking dye. Reaction products characteristic for purified mammalian collagenases, the T_cA and T_cB fragments, are difficult to identify on this autoradiogram. However, by decreasing the polyacrylamide concentration in the gel to 8% and including lysis products obtained from an incubation of purified human fibroblast collagenase as reference proteins, characteristic degradation products representing 75% of the α-chain length (the T_cA fragment) could be identified (Fig. 3). Smaller protein fragments were identifiable on both autoradiograms and appear to be the result of a progressive degradation, possibly caused by less specific proteases. Extracts from ileal tissues show more intermediate lysis products than their corresponding colonic tissues (Figure 2). On the contrary, extracts of the latter tissue produced more smaller fragments, as can be seen on the dye front, suggesting a more extended and faster lysis of the larger intermediates. Extraction buffer alone did not solubilize any collagenous material from the fibrillar gels (results not shown).

Table 5: Effects of inhibitors on extracted collagenolytic activity. Results are expressed as average percentage residual activity ± S.D.

Inhibitor or procedure	Ileal Anastomoses	n	Colonic Anastomoses	n
None	100		100	
Calcium-free buffer	49 ± 9	5	51 ± 13	5
EDTA 120 mM	47 ± 13	4	40 ± 15	4
O-phenanthroline 6 mM	51 ± 10	3	49 ± 9	3
PMSF 2 mM	108 ± 14	3	108	1
Trasylol 100 µg/ml	66	1	72	1
SBTI 100 µg/ml	71	1	102	1
Fetal calf serum 5%	19 ± 6	4	20 ± 4	3
hrTIMP 5U/ml	90 ± 12	5	89 ± 16	5

Finally, we have sought to inhibit the extractable collagenolytic activity by adding several protease inhibitors to the assays. Table 5 summarizes the effects of

proteinase inhibitors on the degradation of reconstituted collagen type I fibrils. The inhibitory effects were similar in both intestinal segments with the exception of SBTI which appeared to be ineffective against the activities extracted from colonic anastomoses. The metalloproteinase inhibitors ethylenediamine-tetraacetic acid (EDTA) and O-phenanthroline partially inhibited the collagenolytic activity in both ileal and colonic extracts, but only when applied in high concentrations. This is due to the fact that the assay buffer contained a relatively high level of calcium ions (50 mM). A comparable inhibitory effect could be achieved by using a calcium-free assay buffer. The serine protease inhibitors showed none (PMSF), minor (SBTI), or moderate (Trasylol) inhibitory capacity. Five percent serum (α2-macroglobulin) proved to be the most powerful inhibitory agent, reducing the degradation of the collagen substrate to 25% or less. The human recombinant tissue inhibitor of metallo-proteinases (hrTIMP) was barely effective against the collagenolytic activity present in the extracts of the rat intestinal anastomoses.

DISCUSSION

Appropriate healing of injured tissues involves the removal of damaged tissue components and the synthesis and deposit of new material. In studies on wound healing, interest is generally focused on the formation of new connective tissue, while the degradative processes leading to matrix depletion receive far less attention. Still, the current view holds that a certain degree of extracellular matrix resorption is an integral part of tissue repair [23]. The strength of intestinal anastomoses depends, during the immediate postoperative period, on the 'suture holding capacity' of the existing collagen fibrils [24] and therefore control of enzyme activities capable of degrading these fibrils should be of particular interest to those who seek ways to improve intestinal healing.

We have shown before, using only soluble type I collagen as a substrate, that extractable anastomotic collagenolytic activity was transiently increased during the first week

after operation [16]. It remains possible that aspecific proteolytic enzymes contribute significantly to the degradation, measured at 37°C, of such a soluble substrate. Therefore, we have extended these observations with measurements on the degradation of fibrillar substrates. For practical purposes, 24-h old anastomoses were used for extraction. The present results confirm the existence of a postoperative increase in collagenolytic activity. The fact that this effect is also apparent with both fibrillar substrates indicates the involvement of true collagenolytic enzymes rather than non-specific proteases. Recent efforts to measure increased collagenase activity in 1-day-old colonic anastomoses failed to yield a significant difference between anastomotic and control tissue [25]. In these experiments, collagenase was measured in media from cultured tissue explants. Apparently, the direct measurement of enzyme activity in tissue extracts, employed by us, is more suitable to demonstrate postoperative changes.

It is noteworthy that only minor differences in activity were found between assays using soluble and fibrillar type I as a substrate. One would expect the soluble substrate to be more easily degraded. An explanation may be that the soluble substrate in fact contained 'diffuse fibrils' [19] since no attempt was made to actually prevent fibrillogenesis during the assay.

Fibrillar type III collagen was also used as a substrate, in an attempt to obtain more specific information on the cellular origin of the collagenolytic activity. If polymorphonuclear leucocytes, known to be present in great numbers at the chosen time point, would be the dominant source of collagenolytic activity, type I collagen should have been degraded far more easily than type III collagen, since the leucocyte collagenase has a significantly increased substrate-specificity for type I collagen [26]. In fact, the assay using type I as a substrate yielded only marginally higher activities than the assay using type III as a substrate. Thus, it is unlikely that polymorphonuclear leucocytes are the primary source for the collagenase activity. Recently, Chowcat et al. [15] have shown that infiltrating cells in the anastomotic region were not immunoreactive against collagenase antibodies. Therefore,

the role of infiltrating neutrophils as a potential source of collagenolytic enzymes remains questionable. Resident fibroblasts could be a source of enzyme activity or degradation may be mediated by enzymes bound extracellularly to matrix constituents. Still, inflammatory cells might actually play an important role as suppliers of collagenase activators, and as regulators of TIMP activity [27]. Furthermore, these cells secrete cytokines: powerful stimulators of fibroblast cell proliferation and cellular production of collagenolytic enzymes.

Loss of collagen fibrillar structures from the anastomotic area is expected to decrease the 'suture holding capacity' of the intestinal wall, increasing the risk for anastomotic failure. It is very interesting to observe that the postoperative increase in collagenolytic activity was significantly higher around colonic than around ileal anastomoses. This result supports the hypothesis that the immediate postoperative collagen degradation is greater in colon than in ileum [13]. In addition, we have found recently that postoperative anastomotic collagen synthesis is delayed in colon as compared to ileum [28]. Together, these phenomena may induce a state of lowered collagen levels in the anastomotic area which is more extensive and longer-lasting in colon than in ileum. This could be one of the reasons that chances for dehiscence are greater in colonic anastomoses and thus explains the higher failure rates of anastomoses in the large bowel.

Chromatography of the products released from a reconstituted collagen type I fibrillar matrix has demonstrated two or more, simultaneously acting, processes. The first process results in the release of intact collagen molecules and its occurrence was demonstrated by the presence of abundant mono-, di- and polymeric collagen chains in the assay supernatants. Both ileal and colonic extracts solubilized intact collagen molecules while buffer alone did not release any collagenous material. Since crosslinks, which hold individual collagen molecules together within fibrils, are mainly found in the telopeptide region [29], primary desintegration of the matrix seems to be focused on the cleavage of these stabilizing structures. True mammalian collagenases have, to our knowledge, never

been reported to be involved in cleavages in the telopeptide regions. Therefore, the involvement of a telopeptidase-like protease or 'cross-linkase'as a destabilizing enzyme seems to be very likely. In recent nomenclature of connective tissue degrading enzymes this activity is classified as matrix metalloproteinase type 4 (MMP-4) [30].

The solubilization of intact collagen molecules is most likely followed by the action of a classical vertebrate collagenase. These enzymes are generally accepted to be, physiologically, the most prominent proteases capable of inducing a primary scission in the tight α-helix domain of a collagen molecule. Demonstration of the presence of the characteristic lysis products of this scission, 25% and 75 % long peptides, is commonly taken as evidence for the presence of a true mammalian collagenase (MMP-1). The difficulties we encountered to identify these primary lysis products are most likely due to the heterogenous composition of the extracts. However, the results shown in Fig. 3 clearly demonstrate the presence of a true mammalian collagenase in the extracts of both ileal and colonic anastomoses.

Modulation of collagen breakdown around intestinal anastomoses should, ideally, affect the rate limiting step in the lysis process. Within the sequence of activities previously mentioned, the true mammalian collagenase represents an important target of for the inhibition of collagen degradation. The tissue inhibitor of metalloproteinases has been shown to be a powerful *in vitro* inhibitor of active collagenase (MMP-1). However, inhibition studies carried out with human recombinant TIMP (hrTIMP) revealed that this inhibitor was hardly effective against the activity extracted from rat intestinal tissue samples. A possible explanation for the lack of inhibitory activity of hrTIMP against our extracts might be a different affinity of hrTIMP for the collagenolytic enzyme(s) synthesized by rat cells. Very recently evidence has been presented for the existence of a second TIMP-like inhibitor (TIMP-2) [31] and it has been proposes that there exists a family of TIMP-related (glyco-)proteins with more or less specific target enzymes within the family of the metalloproteinases. The unexpected lack of inhibitory activity of the hrTIMP

against the rat enzyme may be explained within this perspective. Another explanation might be sought in the fact that rat enzymes differ remarkably in their amino acid composition from the human analogues. Thus, it remains possible that hrTIMP, although not inhibitory against the collagenolytic activity detectable in our rat wound healing model, might be an important agent in preventing excessive collagen degradation in other species, including man. Further experiments are necessary to investigate this possibility.

REFERENCES

1 **Fielding LP., Stewart-Brown S., Blesovsky L., Kearney G.** Anastomotic integrity after operations for large-bowel cancer: A multicentre study. Br. Med. J. 281: 411-414, 1980.

2 **Hesp WLEM., Lubbers EJC., Hendriks T., De Boer HHM.** Anastomotic insufficiency in small bowel surgery. Langenbecks Arch. Chir. 368: 105-111, 1986.

3 **Turunen,MJ and Peltakallio,P.** Surgical results in 657 patients with colorectal cancer. Dis. Colon Rectum 26: 606-612, 1983.

4 **Goligher,JC; Graham,NG; De Dombal,FT.** Anastomotic dehiscence after anterior resection of rectum and sigmoid. Brit. J. Surg. 57: 109-118, 1970.

5 **Halstead WS.** Circular suture of the intestine- an experimental study. Am. J. Med. Sci. 94: 436-461, 1887.

6 **Thomson HJ., Busutill A., Eastwood MA., Smith AN., Elton RA.** The submucosa of the human colon. J. Ultrastruc. Mol. Struc. Res. 96: 22-30, 1986.

7 **Gabella,G.** The cross-ply arrangement of collagen fibers in the submucosa of the mammalian small intestine. Cell Tissue Res. 248: 491-497, 1987.

8 **Hunt,TK** Disorders of wound healing. World J. Surg. 4: 271-277, 1980.

9 **Capodici,C; Muthukumaran,G; Amuroso,MA; Berg, RA.** Activation of neutrophil collagenase by cathepsin G. Inflammation 13: 245-258, 1989.

10 **Hesp WLEM., Hendriks T., Lubbers EJC., De Boer HHM.** Wound healing in the intestinal wall: effects of infection on experimental ileal and colonic anastomoses. Dis. Colon Rectum 27: 462-467, 1984.

11 **Cronin K., Jackson DS., Dunphy JE.** Changing bursting strength and collagen content of the healing colon. Surg. Gynecol. Obstet. 126: 747-753, 1968.

12 **Irvin TT., Hunt TK.** Effect of malnutrition on colonic healing. Ann. Surg. 180: 765-772, 1974.

13 **Hendriks Th., Vereecken THLG., Hesp WLEM., Schillings PHM., Boer de HHM.** Loss of collagen from experimental intestinal anastomoses: early events. Exp. Mol. Pathol. 42: 411-418, 1985.

14 **Hawley PR., Page Faulk W., Hunt TK., Dunphy JE.** Collagenase activity in the gastro-intestinal tract. Brit. J. Surg. 57: 896-900, 1970.
15 **Chowcat NL., Savage FJ., Hembry RM., Boulos PB.** Role of collagenase in colonic anastomoses: a reappraisal. Br. J. Surg. 75: 330-334, 1988.
16 **Van der Stappen JWJ., Hendriks Th., Boer de HHM.** Collagenolytic activity extracted from intestinal anastomoses of the rat. Matrix 9: 238-243, 1989.
17 **Wirl G.**: Extractable collagenase and carcinogenesis of mouse skin. Connect. Tiss. Res. 5: 171-178, 1977.
18 **Miller EJ., Rhodes RK.**: Preparation and characterization of the different types of collagen. Methods in Enzymology 82A: 33-64, 1982.
19 **Cawston TE., Barrett AJ.**: A rapid reproducible assay for collagenase using [1-^{14}C]acetylated collagen. Anal. Biochem. 99: 340-345, 1979.
20 **Johnson-Wint B.** A quantitative collagen film collagenase assay for large numbers of samples. Anal.Biochem. 104: 175-181, 1980.
21 **Laemmli UK., Favre M.** Maturation of the head of Bacteriophage T4 I Dna packaging events. J. Mol. Biol. 80: 575-599, 1973.
22 **Lowry OH., Rosebrough NJ., Farr AL., Randall, R.J.**: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275, 1983.
23 **Eckersley JRT., Dudley HAF.** Wounds and wound healing. Brit. Med. Bull. 44/2: 423-436, 1988.
24 **Högström H., Haglund U., Zederfeldt B.** Beneficial effect of proteinase inhibitors on early breaking strength of intestinal anastomoses. Acta Chir. Scand. 151: 529-532, 1985.
25 **Chowcat NL., Savage FJ., Lewin MR., Boulos PB.** Direct measurement of collagenase in colonic anastomosis. Br. J. Surg. 77: 1284-1287, 1990.
26 **Horwitz AL., Hance AJ., Crystal RG.** Granulocyte collagenase: Selective digestion of type I relative to type III collagen. Proc. Natl. Acad. Sci. USA 74/3: 897-901, 1977.
27 **Okada Y., Watanabe S., Nakanishi I., Kishi J-I., Hayakawa T., Watorek W., Travis J., Nagase H.** Inactivation of tissue inhibitor of metalloproteinases by neutrophil elastase and other serine proteinases. FEBS lett. 229/1: 157-160 1988.
28 **Martens MFWC., Hendriks Th.** Postoperative changes in collagen synthesis in intestinal anastomoses of the rat. Differences between small and large bowel. Gut 32: 1482-1487, 1991.
29 **Weiss JB.** Enzymic degradation of collagen. Int. Rev. Connect. Tissue Res. 7: 101-157, 1976.
30 **Okada Y., Nagase H., Harris ED.** A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. J. Biol. Chem. 261/30: 12425-14255, 1986.
31 **Stetler-Stevenson WG., Krutzsch HC., Liotta LA.** Tissue inhibitor of Metalloproteinase (TIMP-2). J. Biol. Chem. 264/29: 17374-17378, 1989.

CHAPTER 4

COLLAGENASES FROM HUMAN AND RAT SKIN FIBROBLASTS PURIFIED ON A ZINC CHELATING COLUMN REVEAL MARKED DIFFERENCES IN LATENCY AS A RESULT OF SERUM CULTURE CONDITIONS.

JOS W.J. VAN DER STAPPEN, THIJS HENDRIKS AND BEN M. DE MAN.

Int. J. Biochem. Vol **24** No. 5, pp 725-735, 1992

ABSTRACT.

1. Fibroblasts from both human and rat skin were grown in the presence or absence of serum and the collagenase activity in the medium was partially purified on zinc-Sepharose.
2. During chromatography, using a discontinuous elution gradient, the rat collagenase elutes at different pH and ionic strength than the human collagenase. Both latent and active collagenases of both species are retarded by the affinity matrix.
3. Latency of collagenase in media obtained from fibroblast cultures appears to be influenced by the presence of serum in the culture medium.
4. The results demonstrate that collagenases secreted by fibroblasts cultures established from the same tissue but obtained from different species are biochemically diverse and that, within one species, the amount of active enzyme depends on the presence of a serum factor.

INTRODUCTION

Vertebrate collagenases are believed to play an important role in the pathogenesis of a number of connective tissue diseases, in metastasis and growth of tumors and in wound healing, (for reviews see Harris and Cartwright, 1977; Liotta et al., 1986). Since the first isolation of a vertebrate collagenase from the culture medium of the tadpole tailfin by Gross and Lapiere (1962) this class of enzymes has been the subject of extensive research. Study of collagenases in tissue extracts appears to be hardly possible because of the very low concentrations found *in vivo*. Most of the work on vertebrate collagenases has been performed on collagenases isolated from the culture media of an extended number of cell types. Among the cell types capable of producing large amounts of collagenase, fibroblasts (Birkedal-Hansen et al., 1976), macrophages (Welgus et al., 1985), keratinocytes (Lin et al., 1987), polymorphonuclear leucocytes (Hibbs et al., 1984) and tumor cells (Liotta et al., 1986) are the most well known.

Collagenases synthesized by cultured cells are released

into the culture medium largely in an inactive proenzyme form (Bauer et al., 1975). The mechanism of conversion of the inactive precursor to a catalytic form still needs to be elucidated. Activation *in vitro* may be achieved either by proteolytic enzymes such as trypsin and plasmin or by exposure to organomercurials such as aminophenyl mercuric acetate (APMA). Highly purified collagenases have been obtained from concentrated culture medium of rheumatoid synovial tissue (Woolley et al., 1975), human skin fibroblasts (Stricklin et al., 1977), and pig synovial tissue fibroblasts (Cawston and Tyler, 1979) using established chromatographic techniques.

Wound healing in the intestine, and the essential role of the processes of collagen synthesis and degradation, are topics of current research interest in our laboratory. Since the animal model used in these studies is the rat, we are particularly interested to obtain purified collagenase from a rat source. In an effort to purify the collagenase produced by rat skin fibroblasts we observed remarkable differences in the behaviour of the enzyme if subjected to purification schemes previously described for collagenases from other sources. In the present study we describe the partial purification of two collagenases, secreted by rat and human skin fibroblasts, on a zinc-affinity column and demonstrate that the presence of serum in the culture medium influences the enzymatic form of the collagenase secreted.

Abbreviations used: APMA, aminophenyl mercuric acetate; DMEM, Dulbecco's modified Eagle medium; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinases; DMSO, dimethylsulphoxide; SDS-PAGE, sodiumdodecylsulphate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonylfluoride.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following suppliers: Dulbecco's modified Eagle's medium (DMEM) and lactalbumin-hydrolysate were obtained from Gibco Limited, Scotland; Brij 35, phorbol 12-myristate 13-acetate (PMA) from Sigma Chemical Company, U.S.A.; tissue culture plastics from

Intermed NUNC, Denmark; penicillin from Gist-Brocades NV Delft, The Netherlands; streptomycin from Pharmachemie Haarlem, The Netherlands; [^{14}C] acetic anhydride from The Radiochemical Centre, Amersham, Bucks., U.K.; Sepharose 6B from Pharmacia, Woerden, The Netherlands; Spectra/Por 6 dialysis membrane from Spectrum Medical Industries, Los Angeles, U.S.A.; Aqua Luma from Lumac BV, Landgraaf, The Netherlands and the 96 wells high affinity tissue culture plates from Greiner, Alphen a/d Rijn, The Netherlands. The human recombinant tissue inhibitor of metalloproteinases (hrTIMP) was a gift from Dr. G. Murphy, Strangeways Research Laboratories, Cambridge, U.K. All other chemicals were of analytical grade.

Culture conditions

Human fibroblast cultures were initiated from punch biopsies of the back skin of healthy volunteers. The specimens were minced (2x2 mm) in sterile tissue culture plates and overlaid with 5 ml of DMEM, supplemented with 10% fetal calf serum, containing 100 I.E. penicillin and 100 I.E. streptomycin/ml. The cell cultures were maintained in a humidified 5% CO2, 95% air incubator at 37°C. After approximately 14 days, the fibroblasts were harvested from the primary cultures by treatment with 0.05% trypsin and replated. Light microscopy confirmed that fibroblasts were the only cell type present. Established cultures were grown to confluency in large 175-cm_2 tissue culture flasks in 50 ml DMEM containing 10% fetal calf serum and antibiotics and were refed every third or fourth day. All conditioned media were collected at every change (of medium) and neutralized by adding 5 ml of a 1.05 M Tris-HCl buffer, pH 7.6, containing 0.21 M $CaCl_2$ and 1.05% Brij 35 to every 100 ml of culture medium. Neutralized medium was clarified by centrifugation at 10000 x g for 30 min (4°C) and frozen at -30°C until further processing. Cultures reaching confluency were harvested and washed three times with Hanks buffer (without Ca and Mg) to remove all serum remnants. Washed cultures were put through five 24 h cycles of maintenance in serum-free DMEM, containing 50 ng/ml PMA and 0.2% lactalbumin-hydrolysate. After each 24 h cycle the culture

medium was harvested and replenished by fresh serum-free medium. Harvested serum-free medium was treated as mentioned above. After maintenance in serum-free medium, fibroblast cultures were returned to serum-supplemented medium for several days before being recycled for another 5 days in serum-free culture medium.

Rat skin fibroblast cultures were established from the back skin of germ-free male Wistar rats. Culture and harvest conditions were identical to those described for human fibroblasts.

Chromatography

All human and rat serum-supplemented and serum-free media were thawed and pooled, yielding 4 pools of collagenase containing solutions. Each pool was concentrated 10- to 40-fold in an Amicon ultrafiltration cell fitted with a PM 10 membrane. Concentrated media were dialysed against 25 mM sodium borate, pH 8.0, containing 0.15 M NaCl (buffer I). A zinc-affinity column was prepared according to Porath et al. (1975). Briefly, an aqueous solution of zinc chloride (ZnCl2, 1 mg/ml was passed through a column of chelating Sepharose 6B. Saturation with zinc ions was accomplished as soon as sodium bicarbonate precipitated with zinc ions in the eluate. Thirty to one hundred ml of concentrated media were applied to a 25 x 1.6 cm zinc affinity column equilibrated before with the same buffer. This buffer was passed through the column until the absorbance at 280 nm returned to baseline. The column was further eluted in a stepwise manner with the following buffers: (II) 25 mM sodium cacodylate, pH 6.5; (III) 25 mM sodium cacodylate, pH 6.5, plus 0.8 M NaCl; (IV) 50 mM sodium acetate buffer, pH 4.7, plus 0.8 M NaCl; (V) 50 mM EDTA plus 0.5 M NaCl. All of the above buffers contained 0.05% Brij 35 and 1 mM $CaCl_2$. All steps were performed at 4°C.

Binding to gelatin-Sepharose

Fractions, eluted from the zinc-chelating column and containing collagenolytic activity were pooled and analyzed batch-wise for their capacity to bind to gelatin. Pools

enriched in rat or human collagenase (see results section) were dialysed against 20 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$, 0.5 M NaCl and 0.05% Brij 35 (GS-buffer). One ml samples of the dialysed pools were added to a 100 µl gelatin-Sepharose suspension, equilibrated before with the same buffer, and rotated for 4 h at 4°C. Subsequently, the suspension was centrifugated (5 min, 500 x g) and 1 ml supernatants were collected. The gel matrix was washed twice again with 1 ml GS-buffer and 1 ml supernatants were collected. Dissociation of bound material was accomplished by the addition of 500 µl 10% (v/v) dimethyl sulphoxide (DMSO) to the GS-buffer followed by rotation for 2 h at 4°C. After centrifugation 450 µl of the supernatant was collected. The DMSO wash was repeated once. All fractions were analyzed for collagenolytic activity using fibrillar type I collagen as a substrate.

Assay of collagenolytic activity

Collagenase activity was measured by a soluble assay using [^{14}C]-labelled soluble type I collagen and two assays using [^{14}C]-labelled reconstituted type I and type III fibrils, respectively. Type I collagen used in the soluble assay and type III collagen used in the fibrillar assay were purified from pepsin-solubilized fetal calf skin. Type I collagen used in the fibrillar assay was purified from the acid-soluble fraction of fetal calf skin. Purification of the collagens was achieved by differential salt fractionation at neutral pH (Miller and Rhodes, 1982). In order to obtain pure type III collagen the salt fractionation procedure was performed repeatedly, resulting in a purity of at least 95%, as determined by sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; data not shown). The purified collagens were labelled with [1-^{14}C]-acetic anhydride (25 mCi/mmol) according to Cawston and Barrett (1979). Labelled collagens (0.25-0.50 µCi/ml) were dialysed exhaustively against 0.1 M acetic acid using a 50000 D Spectrapor 6 membrane, prior to storage at -30°C.

Soluble assay

Assays were carried out in 5-fold replicates in 1.5 ml microtubes: 50 µl of each column fraction was added to 20 µl of [^{14}C]collagen (1.25 mg/ml) and 200 µl 50 mM Tris-HCl (pH 7.8), containing 50 mM $CaCl_2$ and 0.5 M NaCl; 50 µl of dialysis buffer was added to control tubes. The reaction was allowed to proceed for 4 h at 37°C and subsequently terminated by the addition of 50 µl 50 mM Tris-HCl (pH 7.8), containing 0.2 M EDTA. Since no measures were taken to prevent fibrillogenesis during the incubation period, the soluble substrate may actually have been converted into 'diffuse fibrils' (Cawston and Barrett, 1979). After reincubation at 40°C for 15 min, to allow denaturation of the degradation products, 100 µl of 5 M NaCl/glacial acetic acid (9:1, v/v) was added and the tubes were placed on ice for at least 30 min (Seng et al., 1984). The tubes were centrifuged at 10000 x g for 10 min and 100 µl of the supernatant was mixed with 3 ml of Aqua Luma and counted in a liquid scintillation analyzer.

Fibrillar assays

Collagen type I or III solutions in acetic acid were neutralized by dialysis against 30 mM Tris-HCl (pH 7.3), containing 30 mM $Na_2HPO_4.2H_2O$ and 135 mM NaCl (Williams et al, 1978). Aliquots of 20 µl neutralized collagen solution were applied to each well of a 96 wells high affinity tissue culture plate and allowed to gel for 1 h in a humidified atmosphere at 37°C (Johnson-Wint, 1980). After gelling the plates were air-dried in a laminar flow hood. Dried plates were washed twice with 200 µl of demineralized water to remove remnants of buffer. Plates treated this way and stored at 4°C did not deteriorate for at least 2 weeks. Assays were carried out in triplicate: 50 µl of the column fraction or appropriate column buffer (as a control sample) and 150 µl of 50 mM Tris-HCl (pH 7.8), containing 50 mM $CaCl_2$ and 0.5 M NaCl were applied to each well. The reaction was allowed to proceed for 22 h at 37°C in a 100% humidified atmosphere to avoid evaporation. At the end of the incubation period the whole content of each well was

transferred into microtubes and centrifuged at 10000 x g for 10 min. A 100 µl sample from the supernatants was transferred into scintillation vials and mixed with 3 ml of Aqua Luma and counted in a liquid scintillation analyzer. Since organomercurial compounds are known to activate latent (pro)-collagenase, 1 mM APMA (final concentration) was routinely added to the incubations. In order to estimate the amount of active collagenolytic activity in the column fractions assays were also carried out without APMA.

Specific protease inhibitors were added to the assay buffer in order the characterize the collagenolytic activity. The effects of inhibitors were determined from the residual activities after co-incubation of the extracts with the protease inhibitor. The collagenolytic activity was expressed as µg [^{14}C]-labelled collagen/degraded/h under the conditions stated.

SDS-PAGE

Fibrillar type I assay supernatants obtained after incubation of 75 µl of partially purified collagenase with 225 µl assay buffer and 20 µl [^{14}C]-labelled collagen for 24 h at 22°C were dialysed against demineralized water and lyophilized. Lyophilized material was separated by SDS-PAGE using 10% polyacrylamide according to Laemmli (1970). Protein bands were visualized by standard autoradiography.

RESULTS

Conditioned culture medium, when neutralized and supplemented with 1 mM $CaCl_2$ and 0.05% Brij 35 and stored at -30°C, retained its collagenolytic activity over a period of at least several months. Collagenolytic activities of relevant column fractions were measured in the three assays described above. Activity profiles were similar no matter what substrate or substrate form was used (Figure 1). We observed only minor differences in activity profiles between assays run with a fibrillar substrate and assays run with a soluble substrate. Figure 1 also shows that the soluble type I substrate is more readily degraded than the type I fibrillar substrate. The type III fibrillar substrate is

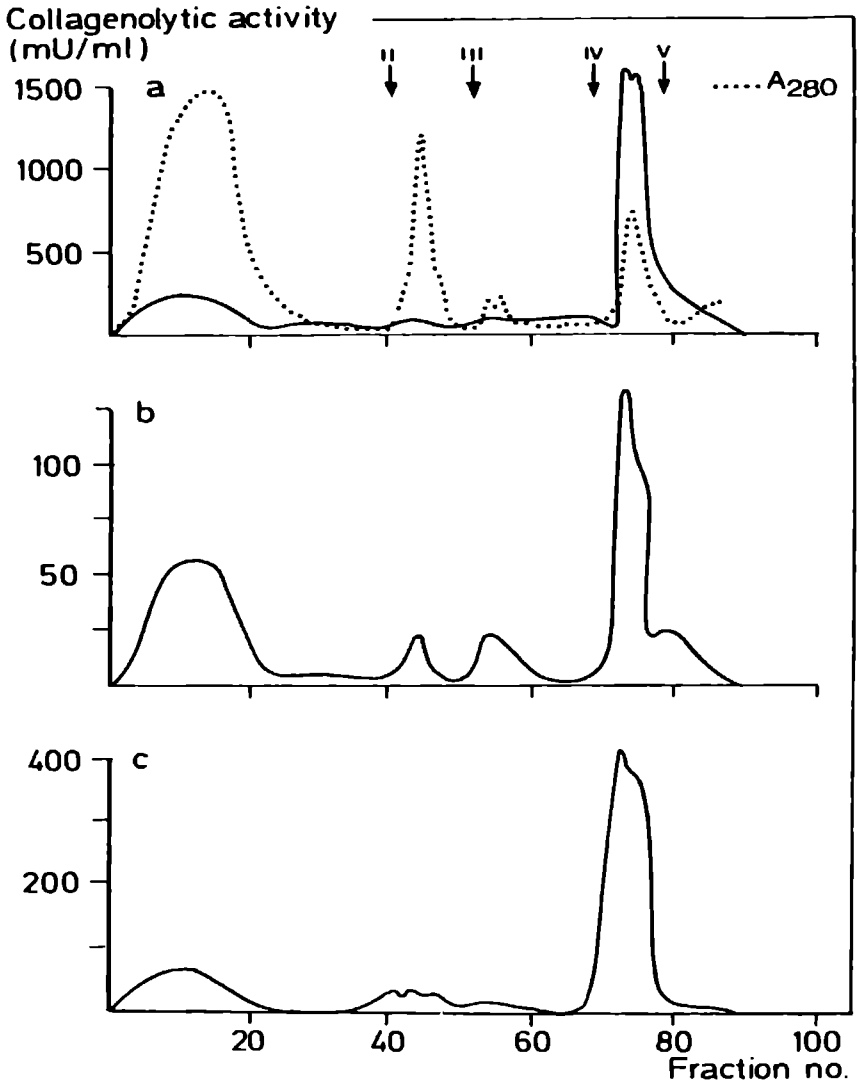

Figure 1. Zinc-chelated column chromatography of conditioned serum-free culture medium of human skin fibroblasts. Sixty ml of concentrated medium was dialysed against buffer I (see Methods section), applied to a 16 x 115 mm column and eluted (30 ml/hr) in a stepwise manner with buffers II-V. Collagenolytic activities in the eluate fractions (5 ml) were assayed using soluble type I (a), fibrillar type I (b) and fibrillar type III (c) collagen as a substrate. All assays represent total collagenolytic activity; 1 mM APMA was included in the assay buffers. Absorbance (A_{280}) is depicted in the upper part of the figure by the dotted line.

more easily degraded than the type I fibrillar substrate. Since the results obtained by the soluble assay did not differ significantly from those obtained by the fibrillar assays but yielded much higher activities, only the column profiles obtained with the soluble assay, in all cases performed both with and without APMA, will be shown in the next section.

Rat skin collagenase, in either latent or active form, was partially purified from fibroblast culture medium. Although unexpected, because of the presence of potent collagenase inhibitors such as α_2-macroglobulin in serum, media from serum-supplemented fibroblast cultures contained significant quantities of latent enzyme (Figure 2). Collagenolytic activity in serum-supplemented conditioned medium of rat fibroblast cultures eluted in two distinct peaks: the first peak eluted when the column was washed with buffer II (see Methods section); an additional peak eluted when the column was washed with the same buffer but with an increased ionic strength (buffer III). If APMA was omitted from the collagenolytic assays it became clear that both collagenase containing peaks represented latent forms of the enzyme: residual activities measured without APMA were about 3% of the total enzymic activity. Eluted fractions were pooled (R+(A), R+(B) and R+(C)) as indicated in Fig. 2. Table 1 gives the recovery and purification factors for the individual pools. The observed increase in total collagenolytic activity is possibly due to underestimation of the activity in the concentrated medium pool. Interference of medium proteins with APMA, leading to insufficient activation of the enzyme in the concentrated pool, or removal of metalloproteinase inhibitors by the chromatographic procedure, may not be excluded either. Collagenolytic activity from the same cells, but produced after switching to serum-free culture conditions, also eluted in two separate peaks from the zinc affinity column (Fig. 3). Apparently, the collagenase activity in these peaks was already in an active form. Since all media were treated identically after harvesting, it seems reasonable to assume that activation of the zymogen took place immediately after secretion. The highest collagenolytic activity was, unlike that in serum supplemented medium, now

found in the first eluted peak. Table 1 shows that a large percentage (73%) of the collagenolytic activity was lost during chromatography. Possibly, the presence of, mainly latent, activity in the unbound fractions contributed considerably to this loss. The peaks obtained after zinc-chelate chromatography were pooled and aliquots were incubated with fibrillar type I as a substrate in order to identify the cleavage products. Figures 4(a) and (b) (lanes C and D) depict the electrophoretograms of these products, visualized by autoradiography, from peak fractions obtained from serum-free and serum-supplemented media, respectively. Collagen degradation products specific for true mammalian collagenase, in particular the 75% TcA fragment, (and 25% TcB cleavage products) were detectable in the assay supernatants. Lanes C and D of Fig. 4 (a) show degradation products of the serum-free pools R-(A) and R-(B), respectively. Lanes C and D of Fig. 4(b) show these products for the serum containing pools R+(A) and R+(C), respectively. Non-specific degradation products of intermediate molecular weight were not observed.

Table 1. Partial purification of collagenase obtained from rat and human fibroblast culture medium.

Source	Total collagenolytic activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Yield (%)	Purification (factor)
Conc. Medium	46800	1002	47	100	1.0
R+(A)	38880	448	87	83	1.9
R+(B)	11490	32	360	25	7.7
R+(C)	11060	4	2953	24	62.8
R+(A/B/C)	61430	484	130	131	2.8
Conc. Medium	64980	56	1162	100	1.0
R-(A)	13300	6	2468	20	2.1
R-(B)	4400	2	1760	7	1.5
(A/B)	17700	8	2213	27	1.9
Conc. Medium	1830	227	8	100	1.0
H+(A)	680	7	97	37	12.1
H+(B)	3000	76	40	164	5.0
H+(A/B)	3680	83	44	201	5.5
Conc. Medium	7140	55	130	100	1.0
H-(B)	9310	17	548	130	4.2

Collagenolytic activity was measured using [^{14}C] labeled reconstituted type I collagen fibrils, incubated with APMA activated samples for 22 h at 37°C. Source codes refer to the bars in the Figs 2, 3, 5 and 6.

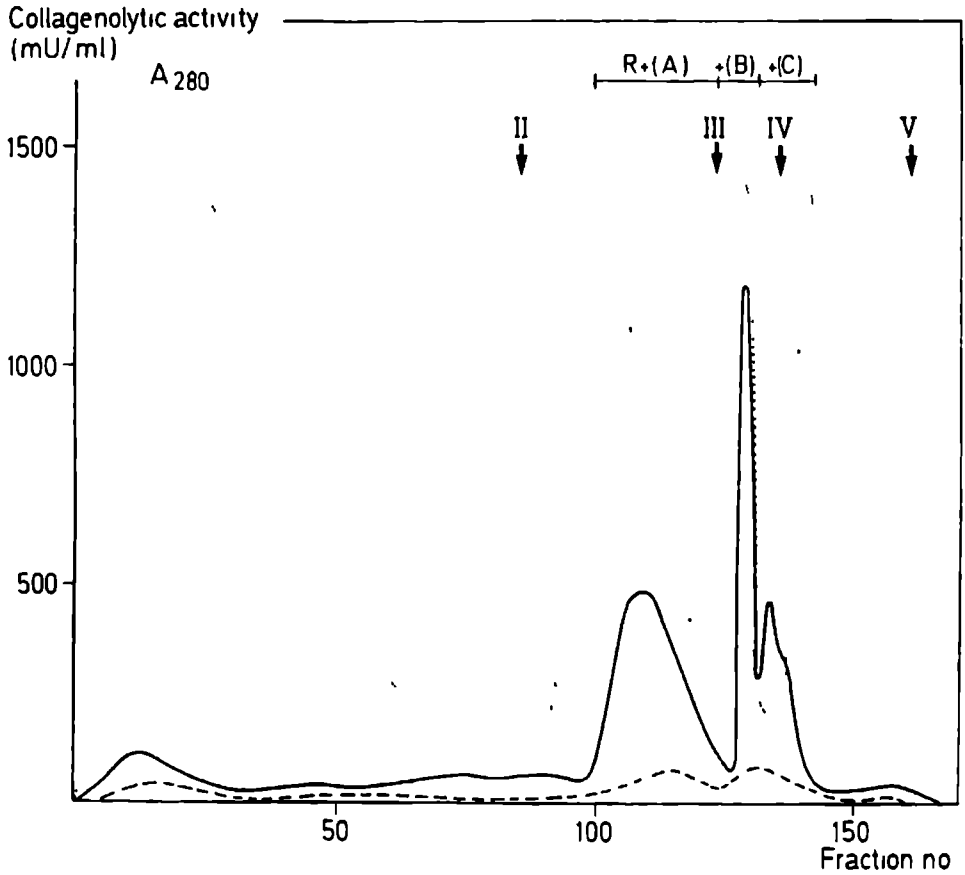

Figure 2. Zinc-chelated affinity chromatography of rat fibroblast collagenase obtained from serum-supplemented culture medium. Sixty ml of concentrated culture medium, dialysed against 25 mM sodium borate, pH 8.0 containing 0.15 M NaCl (Buffer I), was loaded onto a column (25 x 1.6 cm) of chelating Sepharose 6B saturated with ZnCl2. The column was eluted in a stepwise manner (indicated by arrows) with the following buffers, (II) 25 mM sodium cacodylate, pH 6.5, (III) 25 mM sodium cacodylate, pH 6.5 containing 0.8 M NaCl; (IV) 50 mM sodium acetate, pH 4.7, containing 0.8 M NaCl; (V) 50 mM EDTA, plus 0.5 M NaCl adjusted to pH 7.0. All buffers contained 0.05% Brij 35 and 1 mM $CaCl_2$. The column was eluted at a flow rate of 30 ml/hr. 5 ml fractions were collected and assayed for collagenolytic activity using soluble type I collagen as a substrate (——) Total collagenase activity (assayed + APMA); (- - -) active collagenase (assayed without APMA); (.....) absorbance at 280 nm, (|___|) fractions pooled.

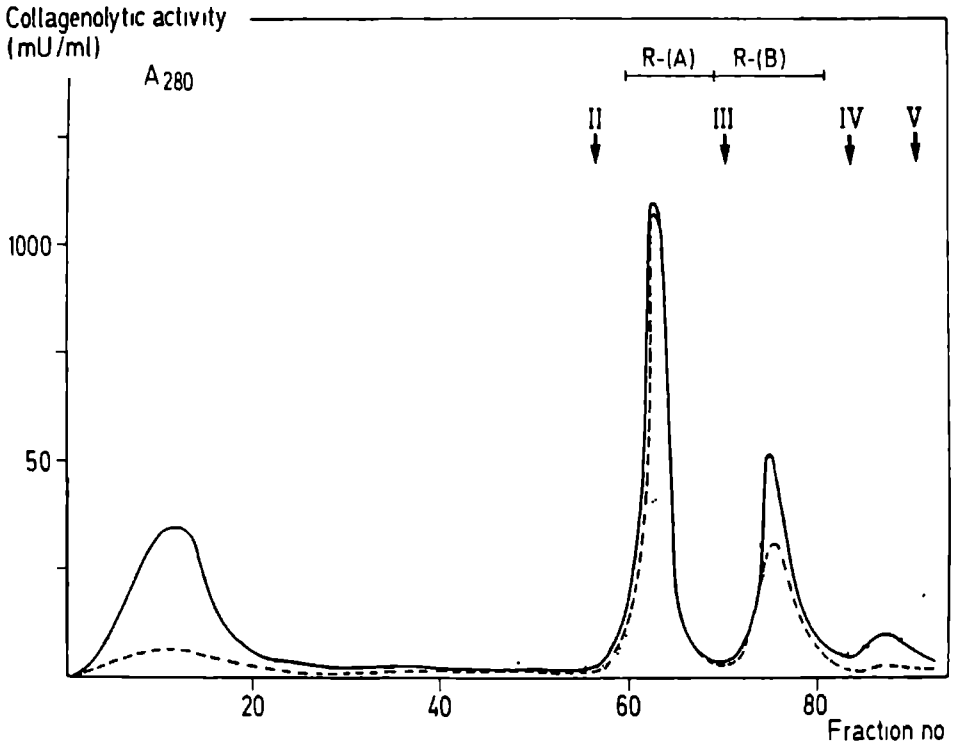

Figure 3. Zinc-chelate affinity chromatography of rat fibroblast collagenase obtained from serum-free culture medium. See legend of Fig. 2 for details.

Human fibroblasts, grown in the presence of serum, secreted collagenolytic activity that eluted in two distinct peaks from a zinc affinity column (Fig. 5). However, the elution profile was different from that obtained by rat fibroblast culture medium (cf. Fig. 2). Maximal collagenolytic activity was now found in the protein peak eluting with buffer IV; a smaller peak eluted with buffer III. The latter fractions appeared to be latent since exclusion of APMA from the assays showed no residual activity. The other peak contained predominantly active collagenase. Collagenolytic activity synthesized by human fibroblast under serum-free conditions produced only one active peak after zinc-chromatography which eluted when the column was washed with the acidic buffer IV (Fig. 6).

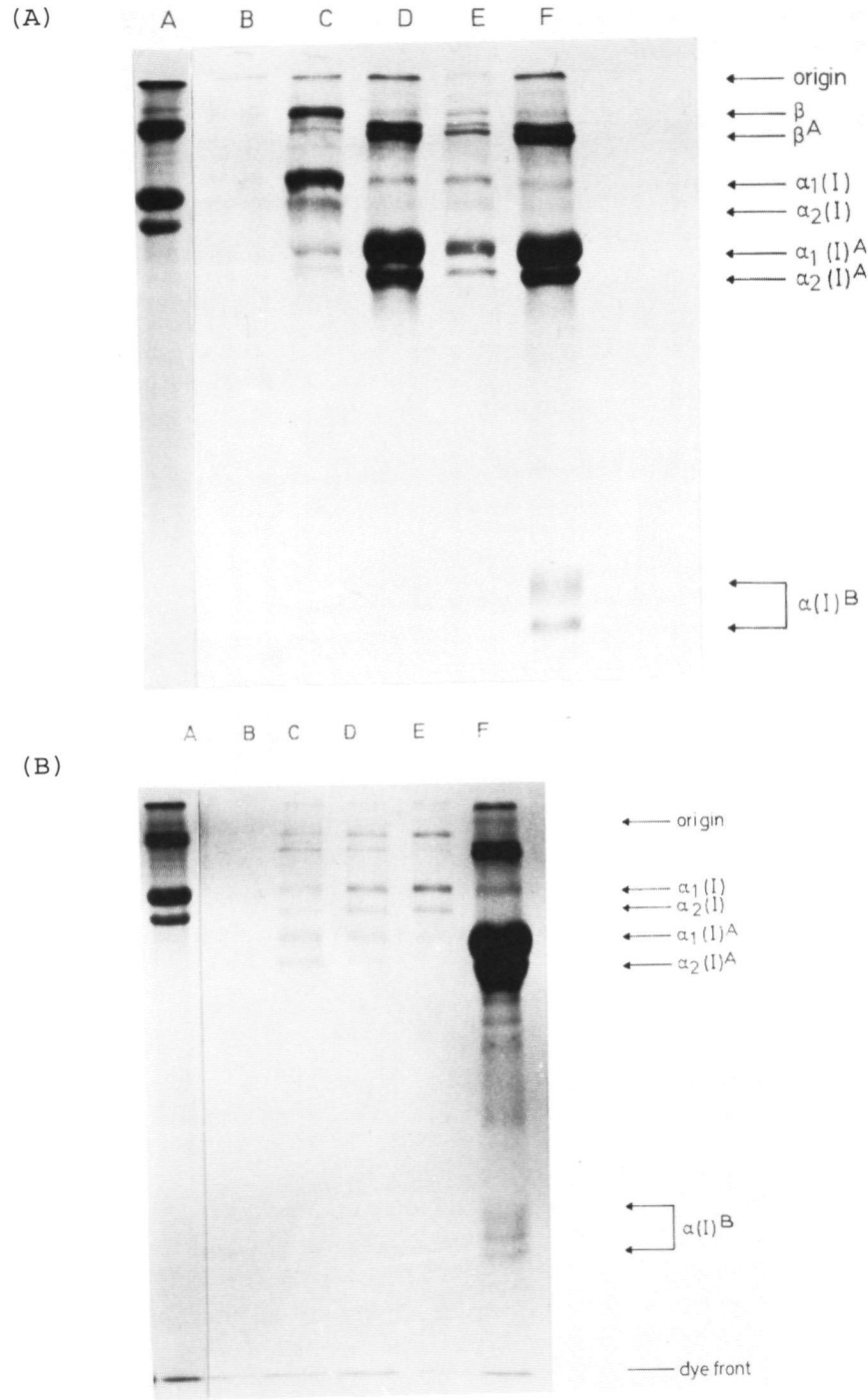
(A)
A
B
C
D
E
F
origin
β
β^A
α1(I)
α2(I)
α1(I)^A
α2(I)^A
α(I)^B
(B)
A
B
C
D
E
F
origin
α1(I)
α2(I)
α1(I)^A
α2(I)^A
α(I)^B
dye front

Figure 4. (Opposite) Cleavage of reconstituted 14C-labeled type I collagen fibrils by human and rat fibroblast collagenase. Assays were carried out at 22°C for 24 hr as described in detail in the Methods section. (a) Samples were taken from pooled fractions after zinc affinity chromatography. Separated media were of serum-free origin. Lane A, type I collagen; lane B, plus assay buffer; lane C, plus a sample from rat pool R-(A); lane D, plus sample from rat pool R-(B); lane E, plus sample from human pool H-(A) and lane F, plus sample from human pool H-(B). (b) Samples were taken from pooled fractions after zinc affinity chromatography. Separated media were of serum-supplemented origin. Lane A, type I collagen; lane B, plus assay buffer; lane C, plus a sample from rat pool R+(A); lane D, plus sample from rat pool R+(C); lane E, plus sample from human pool H+(A) and lane F, plus sample from human pool H+(B).

The efficiency of the purification of the human collagenases is shown in the lower part of Table 1. Total collagenolytic activity exceeded in both cases the amount of activity measured in the original sample. An underestimation of the activity in the concentrated sample as well as the removal of TIMP by column chromatography, are reasonable explanations for this phenomenon.

Collagenase containing fractions were pooled and aliquots were tested for their ability to degrade reconstituted fibrillar type I into specific cleavage fragments. Figure 4(a) and (b) (lanes E and F) confirm that true mammalian collagenase was present in the human eluate pools for, respectively, serum-free (pool H-(B)) and serum-rich (H+(A) and H+(B)) culture conditions. Contrary to the findings in rat material, no degradation products were released by the buffer-III pool (H-(A)) of serum-free cultured human fibroblasts (Fig. 4(a), lane E). However, non-collagenolytic, "telopeptidase-like", activity in the H-(A)pool and all the other pools, appears to be responsible for the release of intact α mono- and dimers. Incubations with buffer alone (lane B of Figs 4(a) and (b)) confirm this interpretation since no collagenous material was detectable in these supernatants.

The pooled fractions of both human and rat sources were incubated batch-wise with a Sepharose-4B coupled gelatin-affinity matrix to examine if collagenase activity would bind to gelatin, thus indicating the capacity to also act as a gelatinase. This experiment shows that part of the collagenase pools obtained from serum-free rat culture

medium indeed bound to the gelatin-matrix: 30% of the R-(A) pool and 49% of the R-(B) pool, respectively. The latter activity represents, according to the specificity of the matrix and its property to degrade the reconstituted fibrillar type I substrate, very likely gelatinase or MMP-2. The pools of collagenolytic activity, obtained from human fibroblasts, cultured both in the presence and absence of serum, or from rat fibroblast cultured in the presence of serum, did not bind to any significant extent to the gelatin matrix.

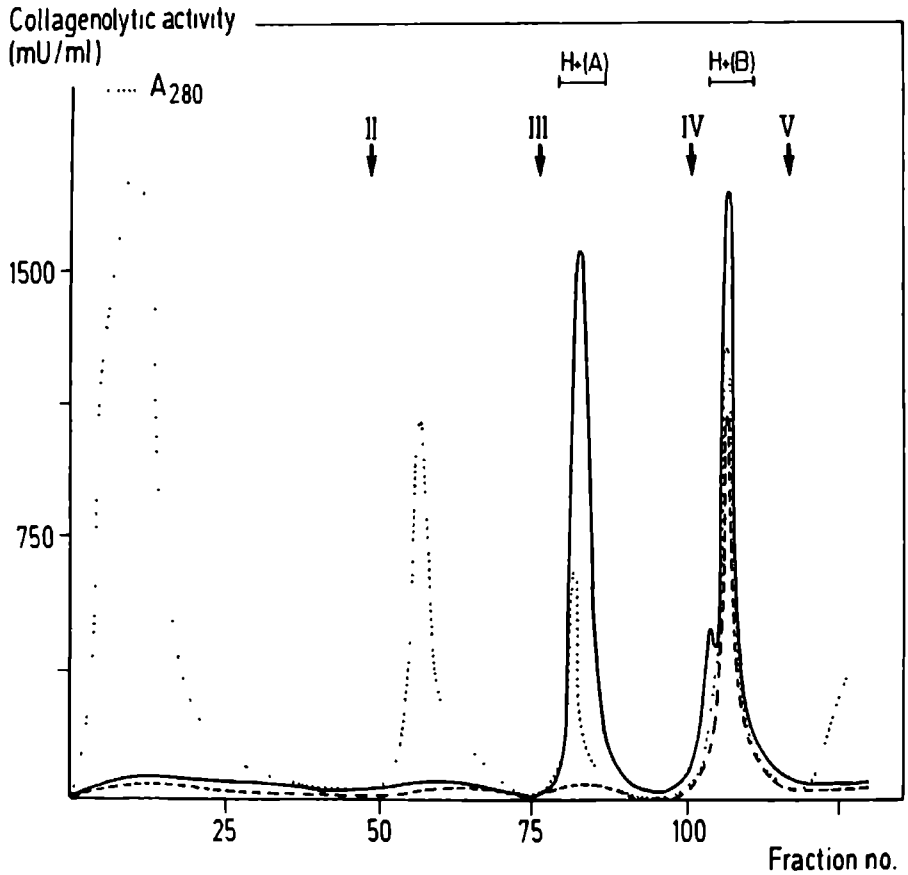

Figure 5: Zinc-chelated affinity chromatography of human fibroblast collagenase obtained from serum-supplemented culture medium. See legend of Fig. 2 for details.

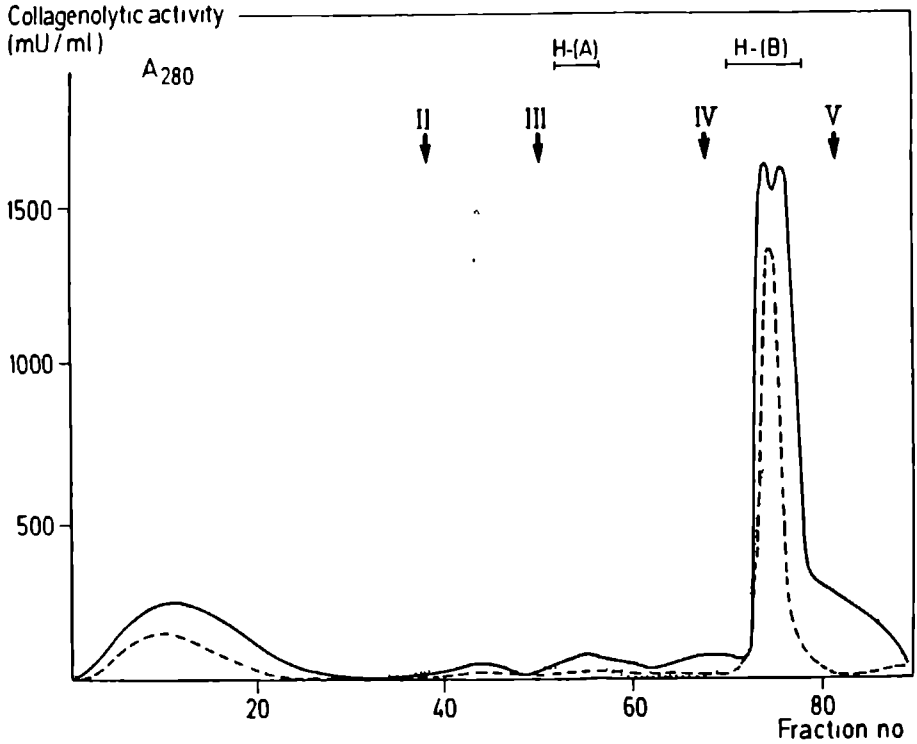

Figure 6. Zinc-chelated affinity chromatography of human fibroblast collagenase obtained from serum-free culture medium. See legend of Fig. 2 for details.

Samples from the eight eluate pools were also incubated with fibrillar type I collagen at 22°C in both the presence and absence of APMA and the supernatants were subjected to SDS-PAGE and autoradiography. Figure 7 depicts the results for the pools obtained from human fibroblasts. Pooled rat eluates showed similar results and are therefore not shown. The amount of degradation products of fibrillar type I collagen released by the action of active and stimulated (latent + active) eluate pools is a clear reflection of the ratio between active and total activity measured by the soluble assay. For example, the amount of collagenous degradation products released from a matrix of reconstituted type I fibrils by a sample taken form the H+(B) pool clearly shows the difference in activity between total (lane C) and

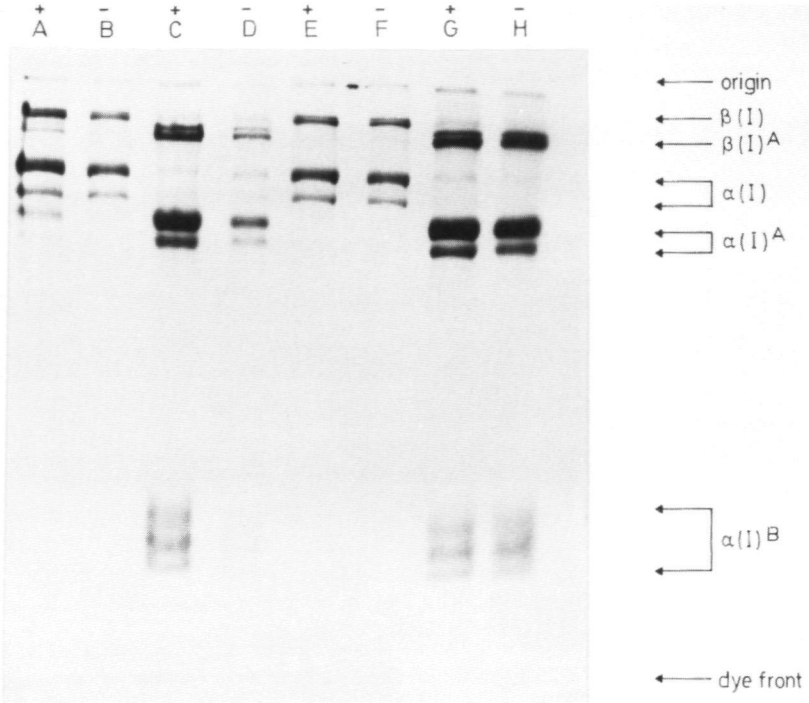

Figure 7. SDS-PAGE of products solubilized by incubation of reconstituted 14C-labeled collagen type I fibrils with partially purified human fibroblast collagenase: Influence of stimulation by APMA. Lanes A, C, E and G: degradation of fibrils by APMA-stimulated collagenase from pools H+(A), H+(B), H-(A) and H-(B), respectively. Lanes B, D, F and H: degradation products obtained after incubation with collagenase from the same pools, in the absence of APMA. Protein bands were visualized by autoradiography.

Table 2. Effect of protease inhibitors on collagenolytic activity of pooled column fractions.

	Serum	Code*	TIMP	EDTA	PMSF
Rat	+	R+(A)	66	48	100
Rat	+	R+(B)	36	37	100
Rat	+	R+(C)	71	87	100
Rat	-	R-(A)	71	58	100
Rat	-	R-(B)	74	100	100
Human	+	H+(A)	47	52	100
Human	+	H+(B)	36	98	100
Human	-	H-(B)	98	31	100

* Code refers to numbered bars in Figs 2, 3, 5 and 6. Final concentrations of inhibitors used are TIMP: 5U/ml, EDTA: 40 mM and PMSF: 2 mM. Activities, measured in the presence of APMA, are expressed as percentage of activity without added inhibitor.

active (lane D) collagenase. This relation is similar to the one exhibited by the corresponding peak fraction and measured by the biochemical assay (cf. Fig. 5). This observation also confirms the reliability of the soluble assay in the screening of the column fractions.

Finally, the pools containing collagenolytic activity were assayed in the presence of some specific proteinase inhibitors using fibrillar type I collagen as a substrate. The results of these experiments are summarized in Table 2. No serine protease activity was detected in the fractions obtained after chromatography. Human recombinant TIMP, added to the assay buffer in the presence of APMA, inhibited the rat and human enzyme pools with the exception of the pool obtained when human serum-free medium was processed. However, the latter sample was strongly inhibited by EDTA. EDTA markedly inhibited most partially purified samples. The differences in inhibitory potential, sometimes found between TIMP and EDTA are apparently due to different inhibitory mechanisms. Complete inhibition of activity could only be achieved by increasing EDTA concentrations (data not shown). We have not investigated the effect of increasing TIMP concentrations because of the limited availability of this compound.

DISCUSSION

Our study unequivocally demonstrates that collagenases secreted by fibroblast cultures established from one tissue but obtained from different species are biochemically diverse. Furthermore, we have presented evidence that, within one single species, the diversity can be induced by the addition of serum to the culture medium.

Fibroblasts, synthesizing true mammalian collagenases (MMP-1) are also capable of a simultaneous production of other members of the matrix metalloproteinase family (e.g. Murphy et al., 1987). Therefore, we have investigated the presence of gelatinase in the pooled collagenolytic fractions by measuring the binding of collagenolytic activity to gelatin. With the exception of the pools obtained from serum-free cultured rat fibroblasts, hardly any binding could be detected. In this case, both the bound

(gelatinase) and unbound (collagenase) fractions degraded reconstituted type I collagen fibrils. Thus, one should be aware that collagenase purified from rat cells may contain considerable gelatinase activity.

Latent human collagenase secreted by fibroblasts cultured with serum appears to become more alkaline compared to the enzyme secreted by cells in a serum-free environment since in the former situation the zymogen partially elutes with buffer of less acidic pH. Human collagenase, both the zymogen and the active enzyme, obtained from serum-free cultures can only be removed from the column with an acidic buffer. A possible explanation of this phenomenon might be the binding of serum proteins to that part of the enzyme that is spliced off during proteolytic activation. Bound serum components might mask acidic residues in this region of the enzyme, thus reducing its binding to the matrix.

The effect of serum proteins on rat collagenase is of a more complex order since two apparently independent effects can be observed. First we have seen that if serum is added to the culture medium only the zymogen is detectable, eluting in two separate fractions from the column, indicative for different binding forces. Secondly, we have found that in serum-free cultures the majority of the enzyme was detectable in an active form. A similar observation was made by Bauer et al. (1972) for the serum-free culture of human skin explants. Simultaneously we observed a diminished affinity for the matrix of a significant quantity of the enzyme since elution was now largely accomplished by a buffer of less ionic strength. The first effect, a lack of activated enzyme, may be explained by an irreversible inhibition of the active enzyme by serum inhibitory components. Active enzyme, complexed by a serum inhibitor, might therefore escape detection by our collagenolytic assays. This explanation might also cover part of the second serum effect mentioned, namely that in the absence of serum two, largely active, peaks were detected. The shift towards less strongly bound enzyme, observed in the elution profile obtained after serum-free culture, may be caused by the same mechanism as proposed for the human enzyme: serum factors masking amino acid side chains, thus increasing or diminishing the ultimate binding of the protein to the

matrix.

Next to the differences observed between active and latent rat and human collagenases we also demonstrated that species derived structural differences are apparent. Preliminary work on the purification of rat collagenase in our laboratory has demonstrated that this enzyme differs in molecular weight and overall charge from the human enzyme. Rat collagenase revealed a clearly higher molecular weight than the human enzyme when eluted over a G-200 gel-filtration matrix. In contrast to the human enzyme which was not retarded by a Sephadex QAE-A50 column the rat enzyme strongly bound to this anion exchange matrix (van der Stappen: unpublished data). The inability of collagenase to bind to a QAE-A50 matrix was also observed by Woolley et al. (1975) for human rheumatoid synovial collagenase and by McCroskery et al. (1975) for a rabbit tumor derived collagenase. The human enzyme was shown to be bound by cation exchange matrices (Stricklin et al., 1977; Wilhelm et al., 1984). Roswit et al. (1983) showed a relative predominance of acidic amino acids in the rat uterine collagenase when compared to the basically charged human enzyme (Woolley et al., 1975), which is consistent with the behavior of both molecules on ion-exchange matrices.

The use of the zinc affinity matrix as a first chromatographic step in a purification procedure is probably a useful tool in separating the zymogen from its active form. Previously described purification methods using zinc-chelating matrices (Cawston and Tyler, 1979; Roswit et al., 1983; Birkedal-Hansen, 1987; Zucker et al., 1988) never mentioned the possibility to discriminate between latent and active enzyme by using this procedure. One might easily overlook the application if this matrix is used further in the purification procedure: earlier reports have shown that certain chromatographic steps preceding zinc affinity chromatography are responsible for activation during purification (Tyler and Cawston, 1980; Stricklin et al., 1983; Zucker et al., 1988). Also, the concentrated medium has been activated prior to any chromatographic step (Tyler and Cawston, 1980; Zucker et al., 1988). Roswit et al. (1988), in their efforts to purify collagenase from serum containing conditioned medium of human smooth muscle cells,

also used a zinc-chelated column as a first chromatograhic step. Since they eluted the collagenase with a glycine gradient, comparison with the present work is impossible. However, it is interesting that they demonstrated that the presence of a serum component was required for the smooth muscle cells to produce collagenase.

In order to verify that true mammalian collagenases, whether as a zymogen or as an active enzyme, have been isolated, SDS gels of enzyme (APMA activated) treated collagen were run to show the presence of T^cA and T_cB fragments. The chromatograms depicted (Fig. 4) confirm the presence of true mammalian collagenases in our preparations and also show that proteases, potentially interfering with the collagenase assays, appear to be absent since additional aspecific cleavage products were not observed. These results are further substantiated by the inhibitor studies. Although the metalloproteinase inhibitors used are not always equally effective, inhibition of collagenolytic activity based on TIMP and/or the metal-chelating properties of EDTA is evident.

The present study has shown ways to obtain partially purified latent collagenase from both human and rat fibroblast cultures simply by using conditioned medium from serum supplemented cultures. The reasonable amounts of partially purified zymogen, obtained in a one step chromatographic procedure, may be very useful in activation and inhibition studies of this metalloproteinase. Activation of latent collagenase is hypothesized (Murphy et al., 1987; He et al., 1989) to be the ultimate step in a cascade of proteolytic events which eventually results in the degradation of connective tissues in normal and pathological conditions. Recent evidence for a protease-involved in *in vivo* activation of latent neutrophil collagenase has been reported by Capodici et al. (1989).

REFERENCES

Bauer EA., Eisen AZ., Jeffrey JJ. Radio-immunoassay of human collagenase. Specificity of the assay and quantative determinations of *in vivo* and *in vitro* human skin collagenase. J. Biol. Sci 247, 6679-6685, 1972.

Bauer, EA.; Stricklin, GP.; Jeffrey, JJ.; Eisen, AZ. Collagenase production by human skin fibroblasts Biochem. Biophys. Res.

Commun. 64/1, 232-240, 1975.
Birkedal-Hansen, H. Catabolism and turnover of collagens: collagenases. In Methods in Enzymology (Edited by Cunningham L.W.) Vol 144, pp 140-171, 1987 Academic Press, New York.
Birkedal-Hansen, H., Cobb, C.M., Taylor, R.E., Fullmer, H.M. Synthesis and release of procollagenase by cultured fibroblasts J. Biol. Chem. 251, 3162-3168, 1976.
Capodici, C.; Muthukumaran, G.; Amoruso, MA.; Berg, RA. Activation of neutrophil collagenase by cathepsin-G Inflammation 13(3), 245-258, 1989.
Cawston TE. and Barrett AJ. A rapid and reproducible assay for collagenase using [1-^{14}C]acetylated collagen. Anal. Biochem. 99, 340-345, 1979
Cawston, TE. and Tyler, JA. Purification of pig synovial collagenase to high specific activity. Biochem. J. 183, 647-656, 1979.
Gross, J. and Lapiere, C. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc. Natl. Acad. Sci. USA 48, 1014-1022, 1962.
Harris, ED. and Cartwright, EC. Mammalian collagenases. In : Proteinases in mammalian cells and tissues. (Edited by Barrett, AJ.) pp 249-283, 1977. North-Holland, Amsterdam,
He, C.; Wilhelm, SM.; Pentland, AP.; Marmer, BL.; Grant, GA.; Eisen, AZ.; Goldberg, GI. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc. Natl. Acad. Sci. USA 86, 2632-2636, 1989.
Hibbs, M.S., Hasty, K.A., Kang A.H. and Mainardi, C.L. Secretion of collagenolytic enzymes by human polymorphonuclear leukocytes. Collagen Rel. Res. 4, 467-477, 1984.
Johnson-Wint, B. A simple vertebrate collagenase assay using soluble radioactive collagen substrate. Anal. Biochem. 104, 175-181, 1980.
Laemmli, UK CLeavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680-685, 1970.
Lin, H.-Y., Wells, B.R., Taylor, R.E., and Birkedal-Hansen, H. Degradation of type I collagen by rat mucosal keratinocytes. Evidence for secretion of a specific epithelial collagenase J. Biol. Chem. 262, 6823-6831, 1987.
Liotta, LA., Rao, CN., Wewer, UM. Biochemical interactions of tumor cells with the basement membrane. Ann. Rev. Biochem. 55, 1037-1057, 1986.
McCroskery, PA.; Richards, JF.; Harris, ED jr. Purification and characterization of a collagenase extracted from rabbit tumours. Biochem. J. 152, 131-142, 1975.
Miller, EJ. and Rhodes, RK. Preparation and characterization of the different types of collagen. In: Methods in Enzymology (Edited by Cunningham L.W. and Frederiksen D.W.) Vol 82, pp 33-64, 1982. Academic Press, New York.
Murphy, G.; Cockett, MI.; Stephens, PE.; Smith, BJ.; Docherty, AJP. Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes. Biochem. J. 248, 265-268, 1987.
Porath, J., Carlsson, J., Olsson, I., and Belfrage, G. Metal chelate affinity chromatography, a new approach to protein fractionation. Nature 258, 598-599, 1975.
Roswit, WT.; Halme, J.; Jeffrey, JJ. Purification and properties of of rat uterine procollagenase. Arch. Biochem. Biophys. 225(1),

285-295, 1983 .
Roswit, WT.; Rifas, L.; Gast, MJ.; Welgus, HG.; Jeffrey, JJ. Purification and characterization of human myometrial smooth muscle collagenase. Arch. Biochem. Biophys. 262(1), 67-75, 1988.
Seng, WL.; Davison, PF.; Kenyan, KR. A simple vertebrate collagenase assay using soluble radioactive collagen substrate. Anal.Biochem. 139, 17-24, 1984.
Stricklin, GP.; Bauer, EA.; Jeffrey, JJ.; Eisen, AZ. Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ cultures. Biochemistry 16(8), 1607-1615, 1977.
Stricklin, GP.; Jeffrey, JJ.; Roswit, WT.; Eisen, AZ. Human skin fibroblast procollagenase: mechanisms of activation by organomercurials and trypsin. Biochemistry 22, 61-68, 1983.
Tyler, JA. and Cawston, TE. Properties of pig synovial collagenase. Biochem. J. 189, 349-357, 1980.
Welgus, H.G., Campbell, E.J., Bar-shavit,Z., Senior, R.M., and Teitelbaum, S.L. Human alveolar macrophages produce a fibroblast-like collagenase and collagenase inhibitor. J. Clin. Invest. 76, 219-224, 1985.
Wilhelm, SM.; Javed, T.; Miller, R. Human gingival fibroblast collagenase-purification and properties of precursor and active forms. Collagen Rel. Res. 4, 129-152, 1984.
Williams, BR.; Gelman, RA.; Poppke, DC.; Piez, KA. Collagen fibril formation. Optimal *in vitro* conditions and preliminary kinetic results. J. Biol. Chem. 253(18), 6578-6585, 1978.
Woolley, DE.; Glanville, RW.; Crossley, MJ.; Evanson, JM. Purification of rheumatoid synovial collagenase and its action on soluble and insoluble collagen Eur. J. Biochem. 54, 611-622, 1975.
Zucker, S.; Turpeenniemi-Hujanen, T.; Wieman, JM.; Lysik, RM. Characterization of a connective tissue degrading metalloproteinase from human small lung cancer cells. Clin. Exp. Metast. 6(5), 363-375, 1988.

CHAPTER 5

CORRELATION BETWEEN COLLAGENOLYTIC ACTIVITY AND GRADE OF HISTOLOGICAL DIFFERENTIATION IN COLORECTAL TUMORS.

Jos W.J. van der Stappen, Thijs Hendriks, Theo Wobbes

Int. J. Cancer: **45**, pp 1071-1078, 1990

SUMMARY

Collagenolytic activity, extracted from 55 tumor and corresponding healthy intestinal control samples, was determined by three different assays using soluble type I and fibrillar type I and III collagen, respectively, as a substrate. The enzyme extracted from tumor digested collagen type I reconstituted fibrils and yielded the three-quarter segments characteristic for the action of one of the matrix metalloproteinases : MMP-1 or mammalian collagenase. Metal-chelating agents such as EDTA and O-phenanthrolin indeed inhibited this activity. Collagenolytic activities were calculated on the basis of wet weight, total DNA and total extracted protein. Correlations were sought between levels of activity and both clinocopathological stage (Dukes' staging) and grade of histological differentiation. In all the assays applied, significant correlations were found between grade of histological differentiation and collagenolytic activity expressed as the tumor/control ratios: poorly differentiated tumors exhibited a higher tumor/control ratio than well-differentiated tumors. Also, tumors penetrating into the serosa showed a higher tumor/control ratio than tumors invading the muscularis propria only. A relation between collagenolytic activity and clinicopathological stage was only observed if activities were calculated on a DNA basis. These results confirm a relation between histological appearance of a tumor and its enzymatic potential to degrade interstitial collagens.

The abbreviations used are: SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis APMA: p-Aminophenylmercuric acetate.

INTRODUCTION.

The invasive and metastatic potential of tumors depends largely on the ability of tumor cells to rearrange and penetrate their connective tissue envelope. The intact extracellular matrix consists of an impenetrable three-dimensional meshwork of collagen fibers, proteoglycans and glycoproteins. Infiltration of this barrier by tumor cells

can only be achieved by partial solubilization of basement membranes and the underlying connective tissue layers. Peptidases produced by tumor cells or by tumor-stimulated connective tissue host cells are held responsible for the initial step in tumor spread by degrading the extracellular matrix that surrounds all organs (Mullins and Rohrlich,1983; Biswas, 1982).

In the investigation of this process, much attention has been paid to the enzymes which are able to degrade the basement membrane which is the first barrier a proliferating tumor cell encounters (Liotta *et al*, 1982). The collagen in basement membranes is almost entirely type IV (Bornstein and Sage, 1980). Penetration of this basement membrane by tumor cells is a prerequisite for metastasizing. Degradation of basement membrane collagen type IV by an enzyme derived from a metastatic murine tumor supports the hypothesis that tumor metastasis proceeds by destruction of extracellular matrix components (Liotta *et al*,1979).

Liotta and co-workers (1980) have shown that highly metastatic tumor cells exhibit *in vitro* an increased capability for synthesizing a type-IV-collagen degrading neutral protease as compared with less agressive tumor cells. They suggested that this enzyme, identified as a metalloproteinase (Liotta *et al*, 1979), might serve as a marker for metastatic potential; this was confirmed by Turpeenniemi- Hujanen *et al*. (1985) and Nakajima *et al*. (1987). Inhibition of type IV collagenase also reduces invasion of tumor cells through reconstituted basement membrane *in vitro* (Reich *et al*.,1988). Indirect *in vivo* evidence for such differentiation in type IV collagenolytic activity can be found in a recent report by Havenith *et al*. (1988), who observed an inverse relation between the amount of basement membrane type IV collagen immunoreactivity and clinico-pathological stage in a series of human colorectal tumors.

Less attention has been paid to the tumor-induced degradation of the interstitial collagens type I and III in the underlying stroma although its role in tumor invasion might be of significant importance. Extracts of both animal and human tumors may contain large amounts of type I and type III collagenase (Hashimoto *et al*.,1973; Steven and

Itzhaki,1977; Biswas *et al.*,1978). Miller *et al.* (1976) purified a type III collagen degrading enzyme from the culture medium of a rabbit V2 carcinoma. Zucker and co-workers (1987) purified a metalloproteinase with activity against both type IV and type I collagen and gelatin from the cytosol of a highly metastatic mouse melanoma. Breast carcinoma cell lines secrete a collagenase which uses both type I and type III collagen as a substrate (Kao and Stern,1986). Tarin *et al.* (1982), using explants of murine mammary tumors, reported a positive correlation between type I collagenase and tumor spread. Cell cultures from metastatic brain tumors produce high levels of type I collagen degrading enzymes while benign meningiomas produce negligible levels of these enzymes. (Halaka *et al.*,1982)

Much of the work reported on correlations between collagenolytic activity and tumor invasion or metastasis has been performed *in vitro*. Few data exist in which the occurrence of such correlations *in vivo* is confirmed, e.g.by comparing the collagenolytic activity in extracts from resected human tumors of varying clinico-pathological stage. For this reason, the present study was designed to determine whether, and to what extent, extracts of colorectal tumor tissue express collagenolytic potential against type I and type III collagen substrates and if there exists any relation to their clinico-pathological stage and grade of histological differentiation.

MATERIALS AND METHODS

Tissue source

Fresh tissue was obtained from 55 patients which were operated upon for colorectal adenocarcinoma in the St.Radboud University Hospital. Representative samples were taken from the luminal side of the tumor and from healthy, tumor-free, intestine at the resection edge. The latter biopsies were composed of mucosa and submucosa only. Specimens were collected within 30 minutes after resection and frozen in liquid nitrogen. Clinicopathological staging was based on Astler-Coller's modification (1954) of Dukes'

classification: A, lesions limited to the mucosa; B1, lesions invading into but not beyond the *muscularis propria* with negative lymph nodes; B2, lesions penetrating beyond the *muscularis propria* into serosa and pericolic fat with negative lymph nodes; C1, lesions invading into but not beyond the *muscularis propria* with positive lymph nodes; C2, lesions extending beyond the *muscularis propria* into serosa and pericolic fat with positive lymph nodes. Tumors which had metastasized to distant organs were categorized as stage D (Turnbull *et al.*,1967). Each tumor was also classified according to its histological differentiation grade : grade I represents a well-differentiated, grade II a moderately-differentiated, and grade III a poorly-differentiated tumor.

Extraction of enzymatic activity

Tissue extracts were prepared essentialy according to Wirl (1977). Briefly, frozen tissue was pulverized in a Braun Microdismembrator. Approximately 100 mg of pulverized tissue was suspended in 2 ml of 10 mM Tris-HCl (pH 7.5), containing 0.4 M sucrose and 5 mM $CaCl_2$ and rotated subsequently for 30 min. After centrifugation at 9000 x g for 15 min, the pellet was washed twice with the same buffer. The resulting tissue pellet was then extracted for 2 h with 5 M urea in 50 mM Tris-HCl (pH 7.5), containing 0.2 M NaCl and 5 mM $CaCl_2$. After centrifugation at 40000 x g for 30 min the supernatant was dialysed for 16 h against the same buffer without urea in order to remove the urea from the extract. All procedures were performed at 4°C. Measurement of collagenolytic activity was performed on extracts immediately after dialysis. The extract, which sometimes became slightly cloudy during dialysis, was routinely shaken to homogeneity immediately before samples were taken.

Assay of collagenolytic activity

Collagenolytic activity in the tumor and control tissue extracts from each patient was determined by measuring the release of radioactive material from ^{14}C labelled collagen type I in solution and from type I and type III

reconstituted fibrils. Type I collagen used in the soluble assay and type III collagen used in the fibrillar assay were purified from pepsin- solubilized fetal calf skin. Type I collagen used in the fibrillar assay was purified from the acid-soluble fraction of fetal calf skin.

Purification of the collagens was achieved by differential salt fractionation at neutral pH (Miller and Rhodes,1982). In order to obtain pure type III collagen the salt fractionation procedure was performed repeatedly, resulting in a purity of at least 95 %, as determined by SDS-PAGE (data not shown).

The purified collagens were labelled with [1-^{14}C]acetic anhydride (25 mCi/mmol; Amersham, UK) according to Cawston and Barrett (1979). Labelled collagens (0.25-0.50 μCi/ml) were dialysed exhaustively against 0.1 M acetic acid using a 50000 D Spectra/Por 6 membrane (Spectrum Medical Industries, Los Angeles, USA), prior to storage at -30°C.

Soluble assay

Assays were carried out in five-fold in 1.5 ml microtubes : 50 μl of extract was added to 20 μl of [^{14}C] collagen (1.25 mg/ml) and 200 μl 50 mM Tris-HCl (pH 7.8), containing 50 mM $CaCl_2$ and 0.5 M NaCl; 50 μl of dialysis buffer were added to control tubes. The reaction was allowed to proceed for 4 h at 37°C and was subsequently terminated by the addition of 50 μl 50 mM Tris-HCl (pH 7.8), containing 0.2 M EDTA. After reincubation at 40°C for 15 min, to allow denaturation of the degradation products, 100 μl of 5 M NaCl/glacial acetic acid (9:1,v/v) was added and the tubes were placed on ice for at least 30 min (Seng *et al*,1984). The tubes were centrifuged at 10000 x g for 10 min and 100 μl of the supernatant was mixed with 3 ml of Aqua Luma (Lumac BV, Landgraaf, The Netherlands) and counted in a liquid scintillation analyzer.

Fibrillar assays

Collagen type I or III solutions in acetic acid were neutralized by dialysis against 30 mM Tris-HCl (pH 7.3), containing 30 mM $Na_2HPO_4.2H_2O$ and 135 mM NaCl (Williams *et*

al, 1978) Aliquots of 20 µl neutralized collagen solution were applied to each well of a 96 wells plastic tissue culture plate (Greiner High Affinity plates ; Greiner, Alphen a/d Rijn, The Netherlands) and allowed to gel for 1 h in a humidified atmosphere at 37°C (Johnson-Wint,1980). After gelling the plates were air-dried in a laminar flow hood. Dried plates were washed twice with 200 µl of demineralized water to remove remnants of buffer. Plates treated this way and stored at 4°C did not deteriorate for at least 2 weeks.

Assays were carried out in triplicate: 50 µl of extract or dialysis buffer (as a control sample) and 150 µl of 50 mM Tris-HCl (pH 7.8),containing 50 mM $CaCl_2$ and 0.5 M NaCl were applied to each well. The reaction was allowed to proceed for 22 h at 37°C in a 100 % humidified atmosphere to avoid evaporation. At the end of the incubation period the whole content of each well was transferred into microtubes and centrifuged at 10000 x g for 10 min. A 100 µl sample from the supernatants was transferred into scintillation vials and mixed with 3 ml of Aqua Luma and counted in a liquid scintillation analyzer. Since organomercurial compounds are known to activate latent (pro)-collagenase (Sellers *et al*,1977), 1 mM APMA (final concentration) was routinely added to the incubations. In order to estimate the amount of active collagenolytic activity, a number of extracts was assayed both in the presence and in the absence of APMA.

Specific protease inhibitors were added to the assay buffer in order to characterize the possible nature and source of the collagenolytic activity. The effects of inhibitors were determined from the residual activities after co-incubation of the extracts with the protease inhibitor.

After subtracting the blank, which routinely constituted less than 6% of total counts present (also in the soluble assay), the collagenolytic activity was expressed as ng [^{14}C]-labelled collagen degraded/h/mg original wet weight, as ng [^{14}C]-collagen degraded/h/g protein extracted and as ng [^{14}]C-collagen degraded /h/µg DNA.

DNA and protein determination

DNA was measured by the method of Burton (1956); 1-3 mg (wet weight) of pulverized tissue was examined. The protein concentration in a 100 µl sample of the extract was measured by the method of Lowry *et al* (1951), using bovine serum albumin as a standard.

SDS-PAGE and autoradiography

Reaction products of tumor and control extracts in a fibril type I assay (run for 24 hr at reduced temperature (22°C) to avoid further degradation of the TcA fragments by non specific proteases in the crude extracts) were pooled, dialysed against demineralized water, freeze-dried and applied to an 8% polyacrylamide gel. Specific collagenase degradation products (T_cA fragments) were visualized by electrophoresis followed by autoradiography using standard procedures. Rainbow protein molecular weight markers ([^{14}C]-labelled) were from Amersham.

RESULTS

The average age of the patients in this study was 65 years (SD : 12.5, range 26-85 years). Clinico-pathological stage and grade of histological differentiation of the tumors involved are summarized in Table 1.

Table 1: Clinico-pathological stage and grade of histological differentiation of the tumor.

	Histological grading					
Dukes' stage	**I**	**I-II**	**II**	**II-III**	**III**	**Total**
A	-	-	1	-	-	1
B1	1	2	5	-	-	8
B2	3	5	12	2	1	23
C1	-	1	2	-	-	3
C2	2	1	2	2	-	7
D	-	1	9	2	1	13
Benign	-	-	-	-	-	1
Total	6	10	31	6	2	56

A majority of the tumors was moderately differentiated

and fell within the Dukes' stage B group. Although Dukes' stage and differentiation grade are both important in predicting survival, an advanced stage did not always correspond with poorly differentiated tissue. The tumors were located in caecum (n=6), colon (n=17), sigmoid (n=21) and rectum (n=11), respectively. The average collagenolytic activities measured in tumor and normal tissue are given in Table 2.

Table 2: Average collagenolytic activities in extracts from tumor and healthy intestinal tissue.

Collagenolytic activity (ng/h/mg wet weight)	Tumor	Control
Type I (soluble)	506 ± 446 (66-2286)	171 ± 81 (48-421)
Type I (fibril)	304 ± 118 (76-541)	172 ± 74 (46-354)
Type III (fibril)	140 ± 56 (56-257)	76 ± 30 (29-158)
DNA (μg/mg wet weight)	8.7 ± 2.8 (3.9-19.3)	5.2 ± 1.9 (2.0-11.3)
Protein (μg/ml extract)	619 ± 214 (196-1085)	353 ± 160 (11-684)

Results are given with SD and (in parentheses) range of observations. Three assays for collagenolytic activity have been employed using soluble type I and fibrillar type I and type III collagen as a substrate.

The urea-mediated extraction from both tumor tissue and healthy intestine yielded detectable activities in all biopsies for the three assays employed. In the presence of APMA, degradation of reconstituted fibrillar type I collagen reached a maximum of 541 ng/h/mg wet weight in the tumor extracts and a maximum of 354 ng/h/mg wet weight in the extracts from healthy tissue. The only benign specimen excised (ID 41) showed activities in the lower range : 108 ng/h/mg wet weight and 102 ng/h/mg wet weight, respectively, for the polypoid and corresponding healthy tissue.

Degradation levels measured by the soluble type I assay were similar, certainly in the normal (sub)mucosa. However,

in tumors some very high activities were observed. The activities measured by the fibrillar type III assay were lower than those observed using type I as a substrate, but still detectable in all cases.

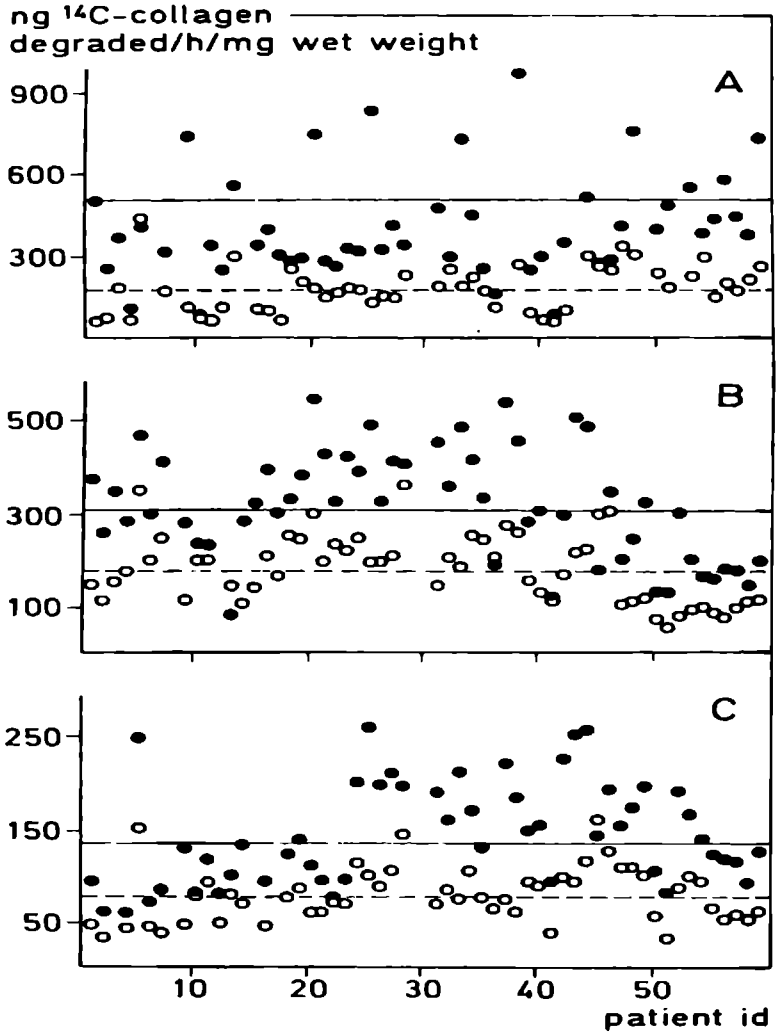

Figure 1: Collagenolytic activity measured in extracts from tumor and healthy intestine. Collagenolytic activity was measured using soluble type I (A), fibril type I (B), and fibril type III (C) collagen as a substrate : 0 tumor ; 0 healthy intestine. Data are given for each patient. ——— average value calculated from tumor extracts, - - - - average value calculated from extracts from healthy intestine.

The collagenolytic activities were significantly higher in the tumor extracts than in the extracts from control specimen, if they were expressed on the basis of wet weight : $p < 0.001$ (Signed rank test) for all the 3 assays. However, the differences remained non-significant if activities were expressed per μg DNA or protein. Figure 1 depicts the collagenolytic activities, expressed on the basis of wet weight, in both tumor and control specimen from all the patients; 54 out of 55 patients showed a higher activity in the tumor extracts, as measured with the soluble type I assay (Fig 1A). If measured by the fibril type I assay 52 out of 55 showed a higher activity in the tumor (Fig 1B) ; in the fibril type III assay 51 out of 52 patients showed a higher activity in the tumor.

Subsequently, we calculated for each patient the tumor/control ratio for the collagenolytic activities, as measured with the 3 different substrates and expressed on the basis of wet weight, protein and DNA. The tumor/control ratios were grouped according to the clinicopathological stage or grade of histological differentiation. This way, significant correlations

Table 3: Correlation between grade of histological differentiation and tumor/control ratio for collagenolytic activities, expressed on the basis of wet weight.

Grade of histological differentiation	(n)	Tumor/control ratio		
		Soluble Type I	Fibril type I	Fibril type III
I	6	2.0 ± 1.0	1.7 ± 0.4[1]	1.5 ± 0.3
I-II	10	2.4 ± 2.0	1.8 ± 0.7	1.6 ± 0.5[1]
II	31	3.2 ± 2.2[1]	1.9 ± 0.6	2.0 ± 0.6[1]
II-III	6	6.0 ± 4.4	2.1 ± 0.5	2.1 ± 0.5
II	2	5.5 ± 3.3	3.2 ± 1.3	2.2 -----[1]
Spearman correlation coefficient		0.419 (54)	0.237 (54)	0.381 (52)
p-value		0.0016	0.0845	0.0054

Activities were measured in tumor and corresponding healthy intestine, using one of three substrates, and expressed on a wet weight basis. Subsequently the tumor/healthy intestine (control) ratios were calculated, the tumors were grouped according to grade of histological differentiation and the Spearman correlation was calculated. Results are given as average ± S.D.
[1] One value from this group missing: average of n-1 measurements.

Table 4: Correlation between grade of histological differentiation and tumor/ control ratio for collagenolytic activities, expressed on basis of protein extracted.

Grade of histological differentiation	(n)	Tumor/control ratio Soluble Type I	Fibril Type I	Fibril Type III
I	6	0.8 ± 0.3	0.8 ± 0.4[1]	0.7 ± 0.4
I-II	10	1.2 ± 1.0	0.9 ± 0.4	0.8 ± 2.3[1]
II	31	1.9 ± 1.4[1]	1.2 ± 0.7	1.3 ± 0.8[1]
II-III	6	4.0 ± 3.9	1.4 ± 0.6	1.4 ± 0.5
III	2	3.0 ± 3.2	1.7 ± 1.6	1.5------[1]
Spearman correlation coefficient		0.508 (54)	0.315 (54)	0.469 (52)
p-value		0.0001	0.0202	0.0005

Activities were measured in tumor and corresponding healthy intestine, using one of three substrates, and expressed on a protein basis. Subsequently the tumor/healthy intestine (control) ratios were calculated, the tumors were grouped according to grade of histological differentiation and the Spearman correlation was calculated. Results are given as average ± S.D.
[1] One value from this group missing: average of n-1 measurements.

Table 5: Correlation between grade of histological differentiation and tumor/control ratio for collagenolytic activities, expressed on the basis of DNA content.

Grade of histological differentiation	(n)	Tumor/control ratio Soluble Type I	Fibril Type I	Fibril Type III
I	5	1.2 ± 0.5	1.1 ± 0.3	1.0 ± 0.3
I-II	9	1.2 ± 1.1	0.8 ± 0.3	0.8 ± 2.9[1]
II	25	2.0 ± 1.4	1.2 ± 0.5	1.2 ± 0.5[1]
II-III	5	3.1 ± 0.9	1.6 ± 0.6	1.8 ± 0.9
III	2	1.9 ± 1.0	1.1 ± 0.4	0.7 -----[1]
Spearman Correlation coefficient		0.447 (46)	0.288 (46)	0.352 (43)
p-value		0.0019	0.0520	0.0206

Activities were measured in tumor and corresponding healthy intestine, using one of three substrates, and expressed on a DNA basis. Subsequently the tumor/healthy intestine (control) ratios were calculated, the tumors were grouped according to grade of histological differentiation and the Spearman correlation was calculated. Results are given as average ± S.D. [1] One value from this group missing: average of n-1 measurements.

were found between tumor/control ratio and grade of histological differentiation. Tables 3,4 and 5 show the average tumor/control ratios for patients grouped according to grade of histological differentiation of the tumor, as calculated from activities expressed on the basis of wet weight, protein and DNA, respectively. With the exception of the tumor/control ratio calculated from the results from the fibrillar type I assay, and expressed on a wet weight basis (Table 3), strong correlations were found between histological differentiation and tumor/ control ratio. That is, the increase in tumor collagenolytic activity with respect to healthy intestine, rises as the tumor becomes less differentiated. This correlation was most significant if collagenolytic activities were used, which had been expressed on a protein basis (Table 4). Also, the increase in tumor/control ratio from grade I to grade III was most explicit if collagenolytic activities were measured using soluble type I collagen as a substrate.

If the results were grouped according to clinico-pathological stage, no such clear correlations were observed. Only if tumor/control ratios were calculated from activities expressed on a DNA basis, and using fibrillar substrates, a significant ($p<0.01$) correlation was found: the tumor/control ratio increased from stage A to D. We also compared the tumor/control ratio in tumors which had invaded only the *muscularis propria* (essentially those classified B1 and C1) with that in tumors which had invaded beyond the *muscularis* and into the serosa (B2, C2 and some with classification D). No differences were observed if the tumor/ control ratio was calculated from activities expressed on the basis of protein. However, if tumor/control ratio's were calculated from activities, expressed on the basis of either wet weight or DNA, significant differences were found from the assays using soluble type I and fibrillar type I collagen substrates (Table 6). Here, tumors which had penetrated further into the intestinal wall, showed a larger increase in collagenolytic activity. This difference remained non-significant for the assays using fibrillar type III collagen as a substrate (results not shown).

Table 6: Tumor/control ratio in relation to degree of tumor invasion.

	Soluble Type I		Fibril Type I	
	wet weight	**DNA**	**wet weight**	**DNA**
invasion into:				
muscularis	2.0 ± 0.9 (11)	1.2 ± 0.8 (8)	1.4 ± 0.5 (11)	0.9 ± 0.5 (8)
serosa	3.9 ± 3.1 (32)	2.1 ± 1.4 (28)	2.1 ± 0.7 (32)	1.2 ± 0.5 (28)
p-value[1]	0.031	0.053	0.004	0.027

Collagenolytic activities were measured in tumor and corresponding healthy intestine, using either soluble or fibrillar type I collagen as a substrate and expressed on the basis of both wet weight and DNA. Subsequently, the tumor/healthy intestine (control) ratios were calculated for the tumors penetrating only the muscularis and those invading also into the serosa. Results are given as average ± SD. [-1] Significance for difference between both groups was calculated using an 1-sided Kruskal-Wallis test.

The effect of APMA on the collagenolytic activity in tumor and control extracts was investigated in the fibrillar type I collagenolytic assay. Table 7 shows that the presence of APMA made no difference on the level of collagenolytic activity, neither in tumor extracts nor in healthy intestine. Inhibition studies revealed that inhibitors specific for metalloproteinases such as EDTA (40 mM) and O-phenanthroline (2 mM) indeed exhibited inhibitory activity against the collagenolytic activity in the tumor extracts: inhibition by 47 % and 25 % respectively was found.

Finally, the products of the degradation of fibrillar type I collagen by tumor extracts and extracts from healthy tissue were visualized after SDS-PAGE and autoradiography (Figure 2). Incubation of the substrate with buffer (lane g) showed no solubilization of collagen chains. However, extracts from both healthy intestine and tumor brought intact α-1 and α-2 monomers into solution. Tumor extracts appeared to degrade the monomers further in a fashion specific for a true mammalian collagenase, as indicated by the presence of 3/4 lenght T_cA fragments. Using this type of gels (8% polyacrylamide), the smaller 1/4 lenght T_cB

fragments migrate in the vicinity of the dye front and are therefore not identifiable.

Table 7: Effect of APMA (1 mM final concentration) on collagenolytic activity using fibrillar type I collagen as substrate.

	- APMA	+ APMA
Tumor	64 ± 22 (18)	67 ± 19 (19)
Healthy intestine	36 ± 9 (19)	33 ± 12 (19)

Extracts were assayed both with and without APMA and average values are given (ng collagen/h/mg wet weight) with S.D. and number of determinations.

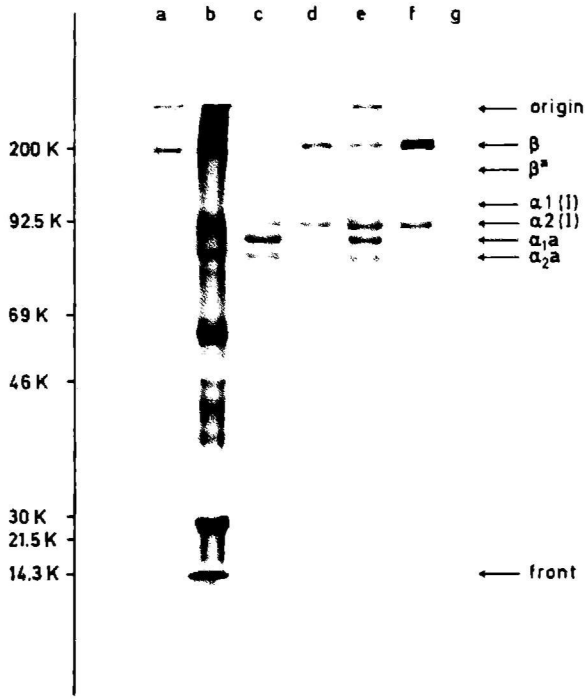

Figure 2: Autoradiography of the cleavage products, separated by SDS-PAGE, from type I fibrillar collagen incubated with extracts from tumor and healthy intestine. Lane a: 14C-labelled type I collagen ; b: molecular weight markers ; c and e :cleavage products solubilized by two different tumor extracts ; d and f: cleavage products solubilized by extracts from the corresponding healthy intestine ; g: dialysed extraction buffer.

DISCUSSION

Three types of assays to detect collagenolytic activity have been applied in this study in order to measure the collagen degradative potential in extracts from tumor and healthy intestine. We have used both soluble type I collagen and reconstituted type I collagen fibrils as a substrate because both forms might actually be present *in vivo*. Purported mechanisms of collagen degradation not only include a direct attack on the collagen fibrils by true collagenases but suppose that the initial step might also consist of the action of broad spectrum proteases on the non-helical terminal regions of the collagen molecule (Burleigh, 1977; Birkedal-Hansen, 1987). The latter proteolytic action might solubilize collagen molecules in a way comparable to the action of pepsin in an acidic environment. For instance, Etherington (1976) has reported the release of alpha chains from insoluble collagen by collagenolytic cathepsin and cathepsin B1 as a result of cleavage of the telopeptide region of the native collagen subunits. Assays performed at physiological temperatures and using soluble collagen substrate are not specific for true mammalian collagenases since non-collagenases are capable of degrading the helical portion of the molecule under these conditions (Birkedal-Hansen, 1987).

Whenever tissue turnover rates are significantly increased (e.g. in wound healing and tumor growth), the newly synthesized connective tissue contains relatively more type III collagen (El-Torky *et al.*, 1985). Since type III collagen appears to be a structural component of the intestinal wall (Graham *et al.*, 1988) we have also measured the degradation of reconstituted fibril type III substrates. Moreover, differences in degradation rates of type I and type III fibrillar substrates might be helpful in the determination of the origin of the enzyme activity. Collagenase originating from polymorphonuclear leucocytes appears to degrade type I collagen more readily than type III collagen (Horwitz *et al.*, 1977) while collagenases from other sources (macrophages, fibroblasts) cleave type III much more easily than type I (Wilhelm *et al.*, 1984; Welgus *et al.*, 1985).

The extraction procedure described yielded extracts in which in all cases, using either of the three substrates, measurable collagenolytic activities could be found. This confirms the efficacy of the method, adopted from Wirl (1977), for investigations on human intestinal tissue. Earlier, we applied this extraction procedure to rat intestine (Van der Stappen *et al.*,1989).

Little is known about collagenolytic activity in human colorectal tumors. After Dresden *et al.* (1972) reported that 66% of colonic adenocarcinomas produced collagenolytic enzymes in organ culture, short reports claimed both a decreased (Sturzaker and Hawley, 1974) and an increased (Horowitz *et al.*, 1987) collagenase activity in mucosal explants of colon carcinomas, as compared with explants of normal intestine. So far, the only more comprehensive study is that reported by Irimura *et al.* (1987), who investigated type I and type IV collagenolytic activity in both extracts and explant media from colorectal carcinomas. They found no difference in activity between tumor and normal mucosa, while our data show, almost without exception (Figure 1), an elevated enzyme activity in tumor extracts. Next to possible differences in biopsy composition, a difference in extraction procedure might be a factor of importance in explaining this discrepancy. Using similar enzyme assays, employing fibrillar type I collagen as a substrate, the average activity in extracts from control specimen was 14 ng/h/mg wet weight in their series against 172 ng/h/mg wet weight in our material. Thus, the extraction procedure used by us appears to be more efficient.

In addition to the existence of increased collagenolytic capacity in tumors, we have been able to demonstrate a strong correlation between grade of histological differentiation and the collagenolytic activity expressed as the tumor/control ratio. Such a correlation has not been found before. Durdey *et al.* (1985), studying the occurrence in human colorectal carcinomas of a group of peptidases potentially involved in collagen degradation, showed significantly elevated peptidase activity in tumor homogenates as compared with homogenates of normal colonic wall; no correlation was found between peptidase activity and either the grade of histological differentiation or

clinico-pathological stage of the tumor. Also, organ cultures of human breast neoplasms produce type I collagenolytic activity. Again, no correlation existed between collagenase secretion and histological type (Ogilvie *et al.*,1985).

Kubochi *et al.* (1986) studied type I and type IV collagenolytic activity in homogenates of lung and gastric tumors in relation to histology and clinico-pathological staging. Their observations appear to support the developing notion that in tumors there exists a clear separation between collagenolytic activities against type IV collagen and type I/III collagen. The first activity is necessary for metastasis, while the latter activity is considered to be essential to local invasion. This is also supported by our finding that tumors which had penetrated into the serosa showed a higher increase in collagenolytic activity (towards type I collagen substrates) than tumors which had invaded only the *muscularis propria* (Table 6). The relative lack of correlation in our study between collagenolytic activity and clinico-pathological stage of the tumor is less surprising.

An extension of the present study with the measurement of type IV collagenolytic activity in colorectal tumor extracts might confirm the results of Havenith *et al.*, (1988), who showed a significant relation between Dukes' staging and the amount of collagen type IV present in the basement membranes of colorectal tumors, although, Irimura *et al.* (1987) could not establish such a correlation.

As in almost all studies preceding ours, the cellular origin of the collagenolytic activity in the tumor tissue can not yet be identified conclusively. Since we have observed only twice as much activity in the type I fibrillar assay compared to the type III assay, the activity does not appear to originate solely from either polymorphonuclear leucocytes or other inflammatory cells: if this were to be the case, a much higher difference in activity against type I and type III might be expected (Horwitz *et al.*, 1977; Wilhelm *et al.*, 1984; Welgus *et al.*, 1985). The methodology used in this investigation does not allow us to attribute the source of enzyme activity either to the tumor cells or to the stromal elements since whole tumor segments presumably containing both cells and stromal elements were

extracted as such.

Immunolocalization studies using antibodies against type IV and/or type I collagenase have reported immunological staining to be present intracellularly (Barsky *et al.*, 1983), in the extracellular space (Huang *et al.*, 1986) and on the surface of cultured tumour cells (Zucker *et al.*, 1987). These contrasting reports clearly indicate that different tumors may exhibit tissue specific actions against host tissues and that it is of great importance to study tumoral actions in their natural environment. Many investigations have been reported on isolated tumor cells maintained in culture systems and thus ignoring the interactions between host and tumor cells. Collagenolytic activity released by tumor cells or by tumor induced host cells might be the result of a cascade of proteinolytic activities finally leading to the activation of latent mammalian collagenase (Murphy *et al.*, 1987; He *et al.*, 1989). Since the proteinase cascade theory appears a very acceptable explanation of *in vivo* collagenase activation, further research should also include measurements of the enzymes potentially involved.

The results from the inhibitor experiments indicate that the extracted enzymes might belong to the group of the metalloproteinases. This conclusion is further supported by our electrophoretic findings, showing the presence of specific collagen degradation products attributed to the action of MMP-1 (mammalian collagenase). Although we use crude extracts of intestinal tissue specimen and the presence of contaminating proteases is to be expected, the electrophoretic pattern clearly proves that classical mammalian collagenases are indeed involved in collagen degradation. The increased collagenolytic activity in the tumor extracts as compared to the control extracts and measured by the enzymatic assays are translated into distinct T_cA fragments on the autoradiogram from tumor supernatants. Cleavage fragments produced by control supernatants are hardly discernible. The autoradiogram further shows that non-specific protease activity might be held responsible for the release of intact collagen molecules from the reconstituted fibrillar meshwork. This "telopeptidase" activity seems to be present in equal

amounts in both tumor and control extracts since monomeric and dimeric collagen chains are found in both supernatants, but not in the assay supernatants of a control sample containing buffer only. These results confirm the hypothesis of Burleigh (1977) regarding the role of broad spectrum proteases in the initial attack of collagenous interstitial structures and support the two-stage mechanism of collagenolysis proposed by this author.

Our findings that all extracted collagenolytic activity was in an active form is in agreement with earlier data on tumoral collagenolytic enzymes (Hashimoto *et al.*, 1973; Yamanishi *et al.*, 1972). However, Steven and Itzhaki (1977) extracted a latent enzyme from rabbit tumor. These observations suggests that different cells secrete different forms of collagenase or that the activation of collagenase is controlled by different inhibitor levels or proteolytic activation.

It is generally accepted that abnormalities of the colonic wall, as observed in *colitis ulcerosa*, and the presence of polyps are often the benign precursors of malignant colorectal tumors. Malignancy might become manifest over periods of more than ten years after the first appearance of abnormality. If were able to predict transformation from, for instance, a polyp to a tumor, this might warrant surgical intervention at an early stage and thus improve patient survival chances. Since collagenolytic activity can be measured in as little as 100 mg of wet weight, it would be possible to perform collagenolytic assays on extracts from benign polyps or biopsies of inflamed colonic wall. Increased collagenolytic activity in extracts of apparently benign tissues could then be used as an indicator for preventive surgical removal of the intestinal segment.

Astler, V.B. and Coller, F.A. The prognostic significance of direct extension of carcinoma of the colon and rectum. Ann. Surg., 139: 326-321, 1954.
Barsky, S.H., Togo, S., Garbisa, S.,and Liotta, L.A. Type IV collagenase immunoreactivity in invasive breast carcinoma. The Lancet, febr 5: 296-297, 1983.
Birkedal-Hansen, H. Catabolism and turnover of collagens Collagenases. Meth. in Enzymol., 144: 140-171, 1987.
Biswas, C., Moran, W.P., Bloch, K.J., and Gross, J. Collagenolytic activity of rabbit V2-carcinoma growing at multiple sites. Biochem. Biophys. Res. Commun., 80: 33-38, 1978.
Biswas, C. Host-tumor cell interactions and collagenase activity In : L.A Liotta and I.R Hart (eds), Tumor invasion and metastasis. Developments in Oncology Vol. 7, pp. 405-425. Martinus Nijhoff Publishers, The Hague/Boston/London, 1982.
Bornstein, P. and Sage,H. Structurally distinct collagen types. Ann. Rev. Biochem., 49: 957-1003, 1980.
Burleigh, M.C. Degradation of collagen by non-specific proteinases. In: A.J. Barrett (edt.), Proteinases in mammalian cells and tissues. pp 285-309, North-Holland, 1977.
Burton, K. A study of the conditions and mechanisms of the phenylamine reaction for the colorimetric estimation of deoxyribonucleic acid.Biochem. J., 62: 15-23, 1956.
Cawston, T.E. and Barrett, A.J. A rapid reproducible assay for collagenase using [1-14C] acetylated collagen Anal. Biochem., 99: 340-345 ,1979.
Dresden, M.H., Heilman, S.A., and Schmidt, J.D. Collagenolytic enzymes in human neoplasms. Cancer Res , 32· 993-996 ,1972.
Durdey, P., Cooper, J.C., Switala, S., King, R.F.G.J., and Williams, N.S. The role of peptidases in cancer of the rectum and sigmoid colon. Br. J. Surg., 72: 378-381, 1985.
El-Torky, M., Giltman, L.I., and Dabbous, M. Kh. Collagens in scar carcinoma of the lung. Am. J. Pathol., 121: 322-326, 1985.
Etherington, D.J. Bovine spleen cathepsin B, and collagenolytic cathepsin. A comparative study of the properties of the two enzymes in the degradation of native collagen. Biochem. J., 153: 199-209, 1976.
Graham, M.F., Diegelmann, R.F., Elson, C.O., Lindblad, W.J., Gotschalk, N., Gay, S., and Gay, R. Collagen content and types in the intestinal strictures of Chron's disease. Gastroenterol., 94: 257-265 , 1988.
Halaka, A.N., Gibson, M., Bird, C.C., Bunning, R.A.D., and Reynolds, J.J. Collagenolytic activity of brain tumors in-vitro. J. Neurol. Neurosurg. Psychiat., 45: 282, 1982.
Hashimoto, K., Yamanishi, Y., Maeyens, E., Dabbous, M.Kh.,and Kanzaki,T. Collagenolytic activities of squamous cell carcinoma of the skin. Cancer Res , 33: 2790-2801, 1973.
Havenith, M.G., Arends, J.W., Simon, R.E.M., Volovics, A., Wiggers, T.,and Bosman, F.T. Type IV collagen immunoreactivity in colorectal cancer.Cancer, 62: 2207-2211, 1988.
He, C., Wilhelm, S.M., Pentland, A.P., Marmer, B.L., Grant, G.A., Eisen, A.Z., and Goldberg, G.I. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc. Natl. Acad. Sci. USA,86: 2632-2636.

Horowitz, I.K., Wellisch, G., Levy, N., and Cohen, E. Collagenase activity in colonic mucosa during inflammatory bowel disease. Clin. Biochem., 20· 79-80, 1987.
Horwitz, A.L., Hance, A.J., and Crystal, R.G. Granulocyte collagenase: Selective digestion of type I relative to type III collagen Proc. Natl. Acad. Sci. USA, 74: 897-901, 1977.
Huang, C.C., Blitzer, A., and Abramson, M. Collagenase in human head and neck tumors and rat tumors and fibroblasts in monolayer cultures Ann.Otol. Rhinol. Laryngol., 95: 158-161, 1986.
Irimura, T., Yamori, T., Bennett, S.C., Ota, D.M., Cleary, K.R. The relationship of collagenolytic activity to stage of human colorectal carcinoma. Int. J. Cancer, 40: 24-31, 1987.
Johnson-Wint, B. A quantitative collagen film collagenase assay for large numbers of samples Anal. Biochem., 104: 175-181, 1980.
Kao, R.T. and Stern, R. Collagenases in human breast carcinoma cell lines.Cancer Res , 46: 1349-1354, 1986.
Kubochi, K., Otani, Y., Maruyama,,K., and Okazaki,I. Development of a direct measurement assay for collagenase against type I and type IV collagens in tissue homogenate and its application in stomach and lung cancers. In : H.Tschesche (edt.), Proteinases in inflammation and tumor invasion, pp 337-356 (Chapter 19). Walter de Gruyter & Co, Berlin/New York,1986
Liotta, L.A., Abe, S., Gehron-Robey, P., Martin, G.R. Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. Proc. Natl. Acad.Sci. USA ,76: 2268-2272, 1979.
Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M., and Shafie,S. Metastatic potential correlates with enzymatic degradation of basement membrane. Nature, 284: 67-68 , 1980.
Liotta, L.A., Garbisa, S., and Tryggvason,K. Biochemical mechanisms involved in tumor cell penetration of the basement membrane. In : L.A Liotta and I.R Hart (eds), Tumor invasion and metastasis. Developments in Oncology Vol. 7, pp. 319-333. Martinus Nijhoff Publishers, The Hague/Boston/London, 1982.
Lowry, O.H., Roseborough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the Folin-phenol reagent. J. Biol. Chem., 193: 265-275, 1951.
Miller, E.J., Harris, E.D. jr, Chung, E., Finch, J.E., McCroskery, P.A., Butler, W.T. Cleavage of type II and III collagens with mammalian collagenase : site of cleavage and primary structure of the NH2-terminal portion of the smaller fragment released from both collagens. Biochemistry 15: 787-792, 1976.
Miller, E.J. and Rhodes, R.K. Preparation and characterization of the different types of collagen. Meth. in Enzymol., 82A: 33-64, 1982.
Mullins, D.E., Rohrlich, S.T. The role of proteinases in cellular invasiveness. Biochim. Biophys. Acta, 695: 177-214, 1983.
Murphy, G., Cockett, M.I., Stephens, P.E., Smith, B.J., and Docherty, A.J.P. Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes. Biochem. J., 248· 265-268, 1987.
Nakajima, M., Welch, D.R., Belloni, P.N., and Nicolson, G.L. Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. Cancer Res , 47: 4869-4876,

1987.
Ogilvie, D.J., Hailey, J.A., Juacaba, S.F., Lee, E.C.G., Tarin, D. Collagenase secretion by human breast neoplasms : a clinicopathologic investigation. J. Natl. Cancer Inst., 74: 19-27, 1985.
Reich, R., Thompson, E.W., Iwamoto, Y., Martin, G.R., Deason, J.R., Fuller, G.C., and Miskin, R. Effects of inhibitor of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastitic cells. Cancer Res., 48: 3307-3312, 1988.
Sellers, A., Cartwright, E., Murphy, G., and Reynolds, J.J. Evidence that latent collagenases are enzyme-inhibitor complexes. Biochem. J., 163: 303-307 ,1977.
Seng, W.L., Davison, P.F., and Kenyan, K.R. A simple vertebrate collagenase assay using soluble radioactive collagen substrate. Anal. Biochem., 139: 17-24, 1984.
Steven, F.S. and Itzhaki, S. Evidence for a latent form of collagenase extracted from rabbit tumor cells. Biochim. Biophys. Acta., 496: 241-246, 1977.
Sturzaker, H.G. and Hawley ,P.R. Collagenase activity and malignant disease. Br. J. Surg., 71: 921-922 , 1974.
Tarin, D., Hoyt, B.J., and Evans, D.J. Correlation of collagenase secretion with metastatic-colonization potential in naturally occuring murine mammary tumours. Br. J. Cancer, 46: 266-278, 1982.
Turnbull, R.B., Kyle, K., Watson, F.R., and Spratt, J. Cancer of the colon : the influence of the no-touch isolation technic on survival rates. Ann.Surg., 166: 420-427, 1967.
Turpeenniemi-Hujanen ,T., Thorgeirsson, U.P., Hart, I.R., Grant, S.S.,and Liotta, L.A. Expression of collagenase IV (Basement Membrane Collagenase) activity in murine tumor cell hybride that differ in metastatic potential. J. Natl. Cancer Inst., 75: 99-103, 1985.
Van der Stappen,J.W.J. , Hendriks, Th., and Boer, H.H.M de. Collagenolyticactivity extracted from intestinal anastomoses of the rat. Matrix, 9: 238-243, 1989.
Welgus, H.G., Campbell, E.J., Bar-Shavit, Z., Senior, R.M., and Teitelbaum, S.L. Human alveolar macrophages produce a fibroblast-like collagenase and collagenase inhibitor. J. Clin. Invest., 76: 219-224, 1985.
Wilhelm, S.M., Javed,T., and Miller, R.L. Human gingival fibroblast collagenase : Purification and properties of precursor and active forms. Collagen Rel. Res., 4: 129-152, 1984.
Williams, B.R., Gelman, R.A., Poppke, D.C., and Piez, K.A. Collagen fibril formation. Optimal *in vitro* conditions and preliminary kinetic results. J. Biol. Chem., 253: 6578-6585, 1978.
Wirl, G. Extractable collagenase and carcinogenesis of the mouse skin. Connec. Tiss. Res., 5: 171-178, 1977.
Yamanishi, Y., Dabbous, M.K., and Hashimoto, K. Effect of collagenolytic activity in basal cell epithelioma of the skin on reconstituted collagen and physical properties and kinetics of the crude enzyme. Cancer Res., 32: 2551-2560, 1972.
Zucker, S., Wieman, J.M., Lysik, R.M., Wilkie, D., Ramamurthy, N.S., Golub, L.M., and Lane, B. Enrichment of collagen and gelatin degrading activities in the plasma membranes of human

cancer cells. Cancer Res., 47: 1608-1614, 1987.
Zucker, S., Turpeenniemi-Hujanen, T., Ramamurthy, N., Wieman, J.,Lysik, R., Gorevic, P., Liotta, L.A., Simon, S.R., and Golub, L.M. Purification and characterization of a connective tissue degrading metalloproteinase from the cytosol of metastatic melanoma cells. Biochem. J., 245: 429-437, 1987.

SUMMARY

An important aspect of a general surgeons daily duties is the performance of gastrointestinal surgery. Surgical treatment of the gut comprises both the removal of diseased tissue and the subsequent restoration of the continuity. To achieve this the surgeon will, in almost all cases, have to construct an end-to-end anastomosis. An important number of gastrointestinal operations involves the resection of tumors. Since most tumors are confined to the more distal areas of the gut suturing will be primarily in the colon and in the rectum.

Despite significantly improved surgical techniques and materials colorectal surgery is still attended by a relatively high incidence of complications due to anastomotic leakage. Since large numbers of microorganisms reside in colon and rectum leakage of colorectal anastomoses easily results in local or, in a more severe case, widespread bacterial infection. The fight against these complications requires not only a prolonged hospital admission but frequently takes a fatal ending. Surprisingly, the occurrence of anastomotic leakage following small bowel surgery is a rare phenomenon.

In order to find ways to prevent anastomotic leakage a sound understanding of the biological processes underlying wound healing in the gut is of paramount importance. An important factor in the healing process that will restore damaged tissues is the biosynthesis of the protein collagen. Collagens define to a large extent the structural integrity of organs and tissues and are found in high concentrations in e.g. bones, tendons, skin and ligaments. Similarly, the strength of the intestinal wall is highly dependent on collagen, which has been deposited in its connective tissue layer, the submucosa. A number of studies has shown that collagen concentrations around an anastomosis decrease notably during the first postoperative period. The loss of anastomotic collagen is hypothesized to be caused by proteolytic enzymes involved in the digestion of damaged tissue during the early phases of wound healing. The strength of the anastomosis during this period is principally dependent on the suture holding capacity of the

undamaged connective tissue (i.e. collagen). The next phase in the healing process is characterized by an increased synthesis of new collagen in the injured area and will eventually restore pre-operative bowel wall strength.

If the healing process deviates from its normal patterns as depicted above, e.g. as the result of extended proteolytic degradation, the regain of bowel wall strength will be seriously delayed and obviously the risk of leakage will increase. The most important enzyme responsible for the degradation of interstitial collagen is mammalian collagenase type 1 (MMP-1: matrix metalloproteinase 1). This enzyme is capable of cleaving peptide bonds within the collagen helix, which is comprised of three polypeptide chains. Under physiological conditions the three peptide chains will uncoil and reveal further cleavage sites to less specific enzymes allowing the completion of collagen breakdown.

Small and large bowel anastomoses exhibit greatly different, clinically manifest, leakage rates. Therefore, a comparison of the healing process in both parts of the gut might reveal mechanistic differences and thus indicate ways to lower the relatively high incidence of anastomotic leakage in the large bowel.

This thesis describes the enzyme-mediated degradation of collagen in experimentally constructed colonic and ileal anastomoses in a rat model. The use of animal models always incorporates a certain risk that animal derived observations can not simply be translated to the human situation. Therefore, we have biochemically compared the collagenases from rat and human sources to expose potential species differences. The human derived enzyme was also studied in a number of tumor biopsies.

Chapter 1 reviews the literature on wound healing in general and more specifically on the healing of bowel anastomoses. The close biological relation between the processes that underlie both wound healing and tumor invasion and metastasis is also explained here. The chapter continues with a comprehensive overview of the structural protein collagen. An understanding of the biosynthesis, molecular structure and the intra- and extracellular degradation of this protein is elemental for scientists

studying wound healing and the tumor associated processes of invasion and metastasis.

Chapter 2 describes the development of a series of techniques required to start the project. Attention is paid to the development of an urea-based extraction procedure for collagenolytic activity from pre- and post-operative bowel segments and the evaluation of referent values such as total protein and DNA in the extracts. Enzymic activities were measured in simultaneously-constructed ileal and colonic anastomoses.

The collagenolytic activity extracted from the anastomotic segments was significantly (even up to 4 times the control values in the colon) increased in both ileal and colonic tissues 12 hours after operation. Increased levels of collagenolytic activity, although gradually decreasing to control values, were present for up to 4 weeks post-operatively. Return to original levels was delayed in colon compared to ileum. These results show that the transient decline in collagen concentration immediately after surgery, correlates with a temporary increase in collagenolytic activity.

In **chapter 3** we have tried to specify the increase in anastomotic collagenolytic activity as observed one day after operation. The increase was significantly more pronounced in large bowel than in small bowel. The use of reconstituted fibrillar collagen type I and III substrates and endopeptidase specific enzyme inhibitors allowed us to discuss the potential contribution of mammalian collagenase to the healing process in more detail. The presence of unique MMP-1 collagen type I cleavage products (the so called TcA and TcB fragments) provided the conclusive evidence for the presence of this enzyme. However, the presence and activity of other enzymes in the extracts was indisputable. The difference in the degradation of type I and type III collagen implicated a minor role for the collagenases derived from inflammatory cells. We postulate that an immobilized, inactive, store of collagenases and related enzymes is continuously present on extracellular matrix components. Upon demand, a swift conversion of these precursors into mature enzymes could be achieved and if necessary be supplemented with newly synthesized material

from local fibroblasts and smooth muscle cells. Inflammatory cells may trigger the activation of the extracellular proteinase reserve. Furthermore, these cells are important to wound healing as they release substantial amounts of chemotactic and growth promoting signals.

The results of our healing studies in the rat would become more impressive if it could be shown that the rat enzyme has some analogy with the human equivalent. Additionally, the availability of highly purified enzyme preparations from rat and human origin would allow the production of specific antibodies. Antibodies against the enzymes would then enable us to perform immunolocalization and enzyme inhibition studies. Unfortunately, at the time this project was in progress, no commercial enzyme preparations were available and so we were forced to try to the purify these enzymes ourselves. In **chapter 4**, we describe how the cultures of human and rat skin fibroblasts provided us with crude enzyme pools. Culture medium, both with and without serum complement, was partially purified over a zinc-chelating matrix using a previously tested protocol. We learned that the rat and human enzyme had very different affinities for the matrix and that latent and active enzyme eluted separately from the column. Latency of the enzymes was dependent on the presence of serum complement in the culture medium. The partially purified rat and human enzyme preparations exhibited very little differences in their capability to degrade fibrillar collagen type I. We concluded that despite the clear differences in molecular structure between the human and rat collagenases, no outspoken differences existed that would invalidate our wound healing conclusions.

There is ample evidence that the biological processes that determine the outcome of wound healing are equally important in the development of tumors. Proteolytic enzymes are key factors in the many physiological and pathological events that take place in a living organism. However, significant differences are found at the level of control of the actual process. A decline in, or occasionally the total lack of control of, regulatory factors can easily develop into a pathological condition. **Chapter 5** describes how collagenolytic enzymes could assist in the spread and

local invasion often observed from tumor cells. Our study aimed to reveal the capacity of extracted colorectal tissues to degrade the interstitial collagen types I and III which, if in effect, would facilitate local invasion. Already, other investigators had shown that enzymes related to collagenase, the gelatinases A and B, were capable of degrading basement membrane type IV collagen and thus allowed the spreading of disseminated cells via the circulation.

The collagenolytic activity was determined in 55 urea-extracted human colorectal cancers. This activity was compared to the activity found in extracts obtained from disease-free tissue resected at some distance from the tumor (usually at the proximal or distal resection edge). The enzyme data were related to the clinico-pathological stage of the tumor (according to the Astler-Coller modification of the Dukes' staging) and the extent of tissue differentiation. A significant correlation existed between the levels of activity and grade of differentiation with higher collagenase activities in less well differentiated tumor biopsies. Patients having a bad prognosis based on the Dukes' staging, i.e. patients with tumors that have invaded the outer layers of the colon wall, contained significantly higher enzyme levels compared to patients with a better prognosis. These results provide further evidence for the hypothesized role of proteolytic enzymes, particularly enzymes involved in the degradation of interstitial collagens, in tumor invasion and metastasis.

Een belangrijk onderdeel van de werkzaamheden van de algemeen chirurg bestaat uit operaties aan het maag-darmkanaal. Chirurgisch ingrijpen aan het maag-darmstelsel heeft tot gevolg dat, na het verwijderen van de door ziekte aangetaste delen, de continuiteit in dit orgaan moet worden hersteld. Om dit te bereiken zal in bijna alle gevallen een darmnaad (anastomose) moeten worden aangelegd. Een belangrijk aantal darmoperaties heeft de verwijdering van tumoren als doel. Aangezien tumoren met name in het distale deel van de darm worden aangetroffen zal het aanleggen van een naad veelal plaatsvinden in het colon en het rectum.

Ondanks sterk verbeterde chirurgische technieken en materialen gaat dikke-darmchirurgie nog steeds gepaard met een relatief hoog aantal complicaties als gevolg van het optreden van naadlekkage. Naadlekkage in het colon en het rectum, waar hoge concentraties van micro-organismen worden aangetroffen, kan leiden tot lokale of, in een ernstig geval, tot gegeneraliseerde bacteriële infecties. De bestrijding van deze complicaties heeft nog (te) vaak een fatale afloop, of gaat op zijn minst gepaard met een langdurige ziekenhuisopname. Opvallend is dat bij operaties aan de dunne darm naadlekkage een relatief zeldzaam fenomeen is.

Het terugdringen van naadlekkages vereist een gedegen begrip van de biologische processen die ten grondslag liggen aan wondgenezing in de darm. Een belangrijk onderdeel in het proces dat leidt tot weefselherstel is de synthese van het eiwit collageen. Collagenen zijn uiterst belangrijke eiwitten wanneer het gaat om de structurele integriteit van een orgaan en worden dan ook massaal aangetroffen in o.a. botten, pezen, huid en ligamenten. De sterkte van de darmwand wordt eveneens bepaald door collagenen die men voornamelijk aantreft in de bindweefsellaag tussen het darmepitheel en de spierlagen, de zogenaamde submucosa. Onderzoek heeft aangetoond dat het collageengehalte in de submucosa ter plekke van de darmnaad postoperatief tijdelijk afneemt. Deze afname wordt toegeschreven aan de activiteit van speciale enzymen belast met het verwijderen van beschadigd weefsel tijdens de eerste fase van het

wondgenezingsproces. Gedurende deze vroege fase wordt de sterkte van een darmnaad voornamelijk bepaald door de sterkte van het weefsel (i.e. collageen) waarin de hechtingen zijn verankerd. De hieropvolgende fase wordt gekarakteriseerd door een verhoogde synthese van nieuw collageen dat geleidelijk de functies van de hechtingen zal gaan overnemen.

Indien het genezingsproces echter niet verloopt zoals hierboven geschetst maar bijvoorbeeld de proteolytische afbraak door nog onverklaarde oorzaak aanhoudt, zal de wond nauwelijks aan sterkte winnen en het risiko op lekkage sterk toenemen. Het veruit belangrijkste proteolytische enzym belast met de afbraak van collageen is vertebraat collagenase (ook wel MMP-1: Matrix Metalloproteinase-1 genoemd). Dit enzym heeft de unieke eigenschap om een eerste, maar voor de verdere collageenafbraak noodzakelijke, klieving aan te brengen in de drie eiwitketens die samen de collageen helix vormen. De klieving van collagenase heeft tot gevolg dat de brokstukken van de helix zich kunnen ontwinden waardoor andere proteolytische enzymen toegang krijgen tot de eiwitketens en volledige afbraak kan plaatsvinden.

Aangezien er duidelijke klinische verschillen bestaan tussen de genezing van naden in de dunne en dikke darm zal het simultaan bestuderen van het genezingsproces in beide darmsegmenten mechanistische verschillen kunnen aantonen. Dergelijk onderzoek levert mogelijk aanknopingspunten op voor therapieën ter voorkoming van naadlekkage in de dikke darm.

Het onderzoek zoals beschreven in dit proefschrift heeft de enzymatische afbraak van collagenen in en rondom naden in dunne en dikke darm van de rat als onderwerp. Het gebruik van een diermodel brengt altijd het risiko met zich mee dat de observaties niet direkt te vertalen zijn naar de mens. Daarom werd eveneens een poging gedaan om humaan en rat collagenase (geproduceerd door huid fibroblasten) te vergelijken.

Tenslotte werd onderzoek verricht naar de activiteit van het enzym collagenase in humane darmtumoren.

In **hoofdstuk 1** wordt een overzicht gegeven van de recente literatuur met betrekking tot de wondgenezing in het

algemeen en meer specifiek met betrekking tot de genezing van darmnaden. De biologisch nauw met elkaar in verband staande processen die plaatsvinden tijdens wondgenezing en de groei en uitzaaing van darmtumoren worden eveneens kort belicht. Het leeuwendeel van dit hoofdstuk behandelt vervolgens het eiwit collageen. Een gedegen inzicht in de synthese, moleculaire opbouw en de intra- en extracellulaire afbraak van dit eiwit is van fundamenteel belang voor onderzoek naar wondgenezing en de metastasering van tumoren. Hoofdstuk 1 bespreekt vervolgens de enzymen die verantwoordelijk zijn voor de afbraak van collageen, de zogenaamde metalloproteinasen. Een literatuuroverzicht dat de biosynthese, secretie, activatie en regulatie van de metalloproteinasen behandelt, besluit de inleiding.

Hoofdstuk 2 beschrijft de ontwikkeling van een aantal laboratoriumtechnieken die nodig waren om het project op gang te brengen. Aandacht wordt besteed aan de extractie van de collagenolytische activiteit uit pre- en post-operatieve rattedarm-segmenten, alsmede de bepaling van referentiewaarden zoals totaal eiwit en DNA gehalte. De enzymactiviteit werd bepaald in extracten van darmnaden die gelijktijding waren geconstrueerd in zowel het ileum als het colon van de rat. De collagenolytische activiteit was 12 uur na de operatie significant hoger (tot zelfs 4x de controle waarden in het colon) in zowel ileum als colon. Gedurende de periode van vier weken na aanleg van de anastomoses nam de verhoogde activiteit geleidelijk weer af tot een normale waarde. Het herstel in het colon verliep veelal trager dan in het ileum. Aangetoond werd dat de tijdelijke afname in collageengehalte direct na operatie parallel loopt met een tijdelijke verhoging in collagenolytische activiteit.

In **hoofdstuk 3** wordt de gevonden postoperatieve verhoging in collagenolytische activiteit, zoals gemeten één dag na operatie nader gespecificeerd. Deze toename was significant hoger in de dikke darm dan in de dunne darm. Het gebruik van fibrillaire substraten van collagen type I en III en specifieke enzymremmers maakten het mogelijk om een uitspraak te doen over de bijdrage van het enzym vertebraat collagenase (MMP-1). De aanwezigheid van MMP-1-specifieke collageen type I splitsings- producten was het ultieme bewijs voor de aanwezigheid van dit enzym. De aanwezigheid

van andere enzymsystemen was eveneens evident. De gemeten verschillen in de afbraak van type I en type III collageen impliceren een ondergeschikte rol voor collagenasen afkomstig van ontstekingscellen. Dit wijst erop dat een geïmmobiliseerde, inactieve, voorraad van collagenasen en aanverwante enzymen continue aanwezig is in de extracellulaire ruimte. Deze voorraad zou op snelle wijze geactiveerd kunnen worden en indien nodig kunnen worden aangevuld met nieuw gesynthetiseerde enzymen afkomstig van lokale cellen zoals fibroblasten en gladde spiercellen. Ontstekingscellen zijn mogelijk betrokken bij de activatie van extracellulaire enzym-precursors. De infiltrerende cellen zijn daarnaast belangrijk als bron van chemotactische en proliferatie-stimulerende signalen.

De resultaten van bovengenoemde wondgenezingsstudies in de rat zouden veel aan waarde kunnen winnen indien aangetoond kon worden dat het ratte-enzym enigzins vergelijkbaar is met het menselijk enzym. De beschikking over gezuiverde enzympreparaten uit rat en mens zou ons tevens de mogelijkheid bieden om, tegen deze enzymen gerichte, antilichamen op te wekken. Deze antistoffen zouden vervolgens gebruikt kunnen worden in immuunlocalisatie en enzym-remmings studies. Het volledig ontbreken van commerciële enzympreparaten noodzaakte ons de isolatie zelf ter hand te nemen. In **hoofdstuk 4** wordt beschreven hoe fibroblasten uit huid van de rat en de mens in kweek werden gebracht en gestimuleerd werden tot secretie van collagenase. Kweekmedium, met en zonder serum-supplementatie, werd verzameld en vervolgens gedeeltelijk gezuiverd over een zink-Sepharose matrix gebruikmakend van een uitgetest protocol. Tijdens de opzuivering bleek dat rat en humaan fibroblast collagenase een afwijkende affiniteit hadden voor de scheidings-matrix. De gebruikte methode bleek eveneens geschikt om actief collagenase te scheiden van latent collagenase. De aanwezigheid van geactiveerd collagenase bleek nauw samen te hangen met de aanwezigheid van serum in het kweekmedium. Het gezuiverde ratte en humane collagenase bleek echter niet te verschillen in de capaciteit om intact collageen type I af te breken. We kunnen dan ook concluderen dat ondanks de waargenomen verschillen in de moleculaire structuur tussen rat en

menselijk collagenase de functie van beide enzymen tijdens wondgenezing niet dramatisch zal verschillen.

Onderzoek heeft uitgewezen dat een aantal biologische processen die belangrijk zijn voor het vlotte verloop van wondgenezing eveneens plaatsvinden tijdens tumorontwikkeling. Proteolytische enzymen bepalen in belangrijke mate het verloop van vele fysiologische en pathologische processen. Het verschil tussen beide fenomenen ligt met name op het terrein van de controle. Het verlies van, of het totaal ontbreken van, controlerende factoren kan een normaal proces ernstig ontregelen en uiteindelijk leiden tot een pathologische conditie. In het afsluitende **hoofdstuk 5** wordt ingegaan op de mogelijke rol van het enzym collagenase in de verspreiding en lokale expansie van tumorcellen. Het onderzoek werd toegespitst op de capaciteit van tumoren om interstitieel collagen type I en III af te breken, een proces dat lokale weefselinvasie zou vergemakkelijken. Verschillende studies hadden reeds aangetoond dat aan collagenase verwante enzymen, de gelatinases A en B, een belangrijke rol spelen in de verspreiding van tumorcellen via de circulatie.

De collagenolytische activiteit werd bepaald in extracten van 55 humane darmtumorbiopten. De activiteit werd vergeleken met de activiteit in de darmwand op enige afstand van het tumorweefsel (meestal het resectievlak). De resulaten van de enzymstudies werden afgezet tegen o.a. de pathologische klassificering van de tumor (volgens Dukes') en de mate van differentiatie van het tumorweefsel. Een significante correlatie tussen mate van differentiatie en enzymactiviteit werd gemeten met hogere collagenase-activiteiten in tumoren die een lage graad van differentiatie ten toon spreidden. Patienten met een tumor die reeds de buitenste lagen van de darmwand had bereikt, bevatten over het algemeen meer enzymactiviteit in het tumorextract dan patienten met een betere prognose. Deze resultaten ondersteunen de hypothese dat proteolytische enzymen, en met name enzymen betrokken bij de afbraak van interstitiële collagenen, een rol spelen bij tumor ingroei en metastasering.

Het tot stand komen van dit proefschrift zou onmogelijk zijn geweest zonder de inspanning van velen. Een aantal van hen wil ik hier dan ook graag bedanken voor hun bijdrage.

Doktor *Thijs Hendriks* die mij op die koude dag in februari 1985, de dag waarop we na meer dan 20 jaar weer eens een Elfstedentocht hadden, meedeelde dat ik mijn universitaire loopbaan in zijn laboratorium mocht beginnen. De wijze waarop jij mij en daaropvolgend nog een groot aantal anderen, veelal Brabanders, succesvol naar de volgende fase in hun carriere hebt begeleid is bewonderenswaardig. Minder succesvol waren je pogingen om de brabantse taal meester te worden en met name de wijze waarop je onder meer het woord "effekes" (helaas niet van toepassing op de tijd die het voltooien van dit proefschrift heeft gehad) uitspreekt zal me lang bijblijven.

André Klompmakers die tijdens de vroege fase van het onderzoek en *Ben de Man* tijdens de lange daaropvolgende periode mij hebben bijgeschoold in een groot aantal biochemische technieken en veel, en ik geef toe *Ben* niet altijd het meest plezierige, werk voor mij hebben uitgevoerd.

Stefan van der Vliet en *Marie-Jose van Hoek* die als HLO-stagiaires veel voorbereidend en uitvoerend werk voor me hebben verricht.

Dr. *Walter Mastboom* voor de onnavolgbare wijze waarop hij mij de microchirurgische technieken heeft bijgebracht die noodzakelijk waren voor het werk beschreven in de hoofdstukken 2 en 3.

De medewerkers van het Centraal Dierenlaboratorium, met name *Jan Koedam, Henny van Wezel, Gerry Grutters* en *Hennie Eikholt* voor de prettige samenwerking en assistentie bij de uitvoering van het dierexperimentele werk.

Dr. *P. van Elteren en T. de Boo* voor de statistische verwerking van de onderzoeksgegevens.

Mijn toenmalige huisgenoten aan de *G. Noodtstraat 76/78*, met wie ik mijn Nijmeegse jaren heb doorgebracht, voor de gezellige sfeer waarin ik daar heb mogen wonen en werken en voor de vriendschappen die deze tijd me heeft opgeleverd.

Mijn en Karin's ouders wil ik bedanken voor hun getoonde steun en interesse in mijn werk.

Last, but definitely not least, *Karin Kusters* die al meer dan anderhalf decenium mijn steun, toeverlaat en lief is en gedurende al deze jaren van studie en promotie er voor gezorgd heeft dat er nog een leven na het werk was. Ik hoop dat de geografische barriere die ons tijdelijk heeft gescheiden in de nabije toekomst zal verdwijnen waardoor ik wat vaker bij je kan zijn.

CURRICULUM VITAE

Jos van der Stappen werd geboren op 23 juni 1958 te Heesch. In 1977 behaalde hij het Atheneum B diploma aan het Titus Brandsma Lyceum te Oss. In hetzelfde jaar werd aangevangen met de studie Biologie aan de Katholieke Universiteit Nijmegen. In januari 1985 werd het doctoraal examen Biologie afgelegd met als hoofdvak Ontwikkelings-biologie der Dieren (o.l.v. Dr. Jan-Erik M. van Deynen) en als bijvakken Experimentele Neurologie (o.l.v. Dr. Ron A. Wevers) en Biochemie (o.l.v. Dr. Fons J.M. Vermorken). Vanaf april 1985 tot april 1989 was hij als wetenschappelijk medewerker verbonden aan het Research laboratorium van de afdeling Algemene Heelkunde van het Sint Radboudziekenhuis te Nijmegen. Tijdens deze periode werd onder leiding van Dr. Thijs Hendriks het onderzoek verricht waarvan de resultaten in dit proefschrift zijn beschreven. Van maart 1990 tot juni 1993 was hij werkzaam binnen de CRC Colorectal Tumour Biology Research Group (Prof. Chris Paraskeva) Department of Pathology and Microbiology University of Bristol, England. Tijdens deze periode werd in samenwerking met Dr. Rose A. Maciewicz (Collagen and Muscle Research Group, University of Bristol, Langford, England) onderzoek verricht naar de rol van de lysosomale enzymen cathepsine B en L in de metastasering van darmtumoren. Momenteel is hij werkzaam in het Department of Medicine, University of Bristol, England, waar hij in samenwerking met Dr. Jeff P.M. Holly de rol van proteolytische enzymen in de regulatie van insulin-like growth factor binding proteins en de effecten hiervan op de groei van (darm)tumoren bestudeert.