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COLLAGEN SYNTHESIS IN THE INTACT AND ANASTOMOSED INTESTINE

STUDIES IN TISSUE EXPLANTS AND CELL CULTURES

MICHAEL MARTENS

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION:

Wound healing is one of the most important features of practical surgery. Whether intentional wounding as in elective surgery or accidental wounding as in traumatology is concerned, healing is the ultimate goal. The healing of intestinal anastomoses remains a topic of ongoing research interest, because clinical practice shows it to be disturbed rather frequently. As a consequence, leakage may occur with concomitant morbidity and even mortality. The occurrence of anastomotic leakages appears unavoidable in large bowel surgery; this seems to be in contrast to the outcome of surgery in the small bowel where the complication has hardly been mentioned [1]. Leakage rates reported for large bowel anastomoses differ enormously, ranging between 0 and 35%. The outcome is clearly dependent on the surgeon performing the operation [2]. Healing of colonic anastomoses may be compromised by a multitude of factors, which often go together, increasing the risk for anastomotic failure. Conditions like shock, local infection, advanced age, malnutrition and radiation therapy have been shown to exert a negative effect on anastomotic wound healing [3-5], thereby increasing the role for anastomotic failure to such extent that the surgeon has to avoid construction of a primary anastomosis. Thus, research on wound healing in the intestine remains much needed: its crucial processes have to be characterized in order to understand the mechanisms underlying disturbed repair and to find ways to minimize anastomotic leakage rates, particularly in high risk situations.

THE HEALING PROCESS:

In order to understand mechanisms whereby anastomotic repair may be impaired, knowledge about the processes involved in undisturbed wound healing is essential. Almost all knowledge on the sequence of events during healing of soft tissues is derived from observations in dermal tissue [6]. The infliction of an injury sets off a complex of processes in the organism which are all directed towards the final purpose of repair, healing of the wound. Wound repair is an extremely complex, dynamic process, during which catabolic and anabolic phases can be observed and which comprises cellular, biochemical, spatial and temporal aspects. A description of

these aspects is only possible if one takes the trouble to divide them into their single elements.

During the early inflammatory phase, disruption of blood vessel results in extravasation of blood components and concomitant platelet aggregation, blood coagulation, and generation of bradykinin and complement-derived anaphylatoxins. Activated platelets also release a cascade of biologically active substances, including a series of molecules that promote cell migration and growth into the injury site [7-9].

Neutrophils are the first cells to infiltrate the area of inflammation, shortly, or simultaneously, followed by monocytes [10]. The function of the neutrophils in the early inflammatory phase is to eliminate contaminating bacteria. The influx of monocytes and their maturation to macrophages seems critical to the initiation of tissue repair [11]. The macrophage also plays an important position in the transition between wound inflammation and the second phase of wound repair, the formation of granulation tissue [12]. Granulation tissue consists of a dense population of macrophages, fibroblasts and neovasculature embedded in a loose matrix of collagen, fibronectin and hyaluronic acid. As fibroblasts proliferate and migrate into the wound area, display a change of phenotype which permitts cell motility and concomitant deposition of the loose extracellular matrix [13]. The fibroblasts also arrange themselves and their newly deposited matrix along the radial axis of the wound, forming cell-cell and cell-matrix interactions and thereby generating a collective tension that results in wound contraction [14].

The third and final phase of wound healing is matrix remodeling. As with all of these phases, this phase overlaps with the previous one. In fact matrix production and remodeling begins simultaneously with granulation tissue formation. Nevertheless, in months following the matrix is constantly altered, starting with a relatively rapid elimination of most of the fibronectin from the matrix and with the slow accumulation of large bundles of type I collagen that provide the residual scar with increasing tensile strength [9].

In studies on wound repair, which involves reconstitution and remodeling of extracellular matrix, attention is mainly focused on the behavior of collagen, presumably since this class of proteins is thought to be primarily responsible for wound strength [15]. Collagen remodeling during scar formation is dependent on both

continued collagen synthesis and collagen catabolism. The degradation of wound collagen is initiated by a variety of collagenolytic enzymes from granulocytes [16], macrophages [17], epidermal cells and fibroblasts [18]. The exact regulation of collagen synthesis is achieved by the interaction of fibroblasts with the surrounding extracellular matrix as well as by cytokines released from platelets, endothelial cells and inflammatory cells [19]. The primary collagen types synthesized in the wound area are interstitial type I and III collagen [20]. Normal mammalian skin contains approximately 80-90% type I collagen and 10-20% type III [21]. It has been postulated that during the course of wound healing the relative ratio of type III to type I collagen synthesis changes [22].

Histological observations confirm that this overall sequence also occurs in the intestinal wall [23-25] and one would certainly like to think that all wounds heal by common mechanisms [26]. However, various authors have expressed the necessity to exert caution in extrapolating the results from research on skin repair to the healing of other soft tissues [27-29]. In fact, it has been shown that significant differences exist between healing in the gastrointestinal tract and skin [30]. Thus if one aims to improve anastomotic healing in the intestine, research for this purpose should be performed on this particular tissue.

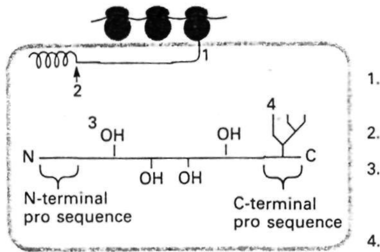
COLLAGEN:

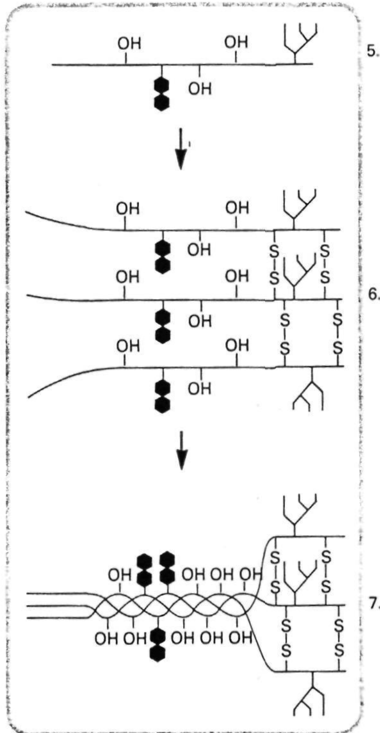
Collagen is the predominant structural protein in all connective tissues of the mammalian organism, including the skin and intestine. Many types of collagen (at least 12; see table 1) have been isolated and characterized, but they all share one feature in common: they are constructed of three polypeptide chains arranged in ropelike triple-helical formation. This triple-helical structure, which confers upon collagen its biological property of high tensile strength, also provides the molecule with an extraordinary degree of resistance to degradation by proteolytic activities. Like other secreted proteins, collagen molecules are synthesized in a precursor form (procollagen) and subsequently cleaved to smaller molecules before aggregating to form collagen fibrils.

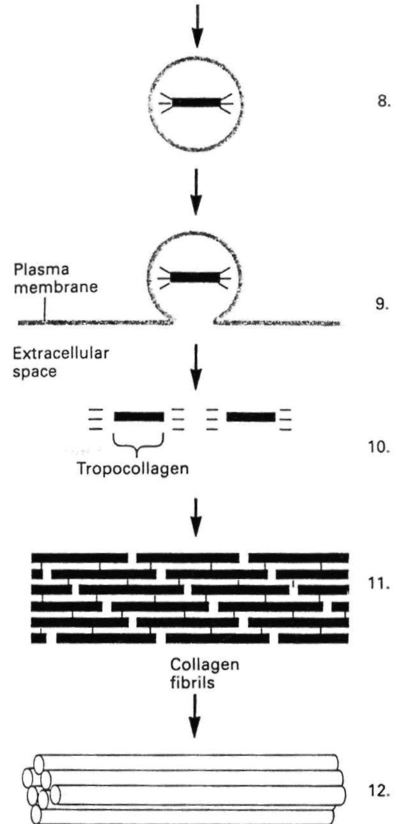
Table 1: Collagen types (this table is still expanding)

collagen type	chains	localization
I	$\alpha1(I)$; $\alpha2(I)$	intestine, skin, tendon, muscle, bone,
II	$\alpha1(II)$	cartilage, vitreous humor
III	$\alpha1(III)$	similar to type I
IV	$\alpha1(IV)$; $\alpha2(IV)$	basement membranes
V	$\alpha1(V)$; $\alpha2(V)$; $\alpha3(V)$	intestine and interstitial tissues
VI	$\alpha1(VI)$; $\alpha2(VI)$; $\alpha3(VI)$	interstitial tissues
VII	$\alpha1(VII)$	anchoring fibrils
VIII	$\alpha1(VIII)$	some endothelial cells
IX	$\alpha1(IX)$; $\alpha2(IX)$; $\alpha3(IX)$	cartilage
X	$\alpha1(X)$	hypertrophic and mineralizing cartilage
XI	$\alpha1(XI)$; $\alpha2(XI)$; $\alpha3(XI)$	cartilage
XII	$\alpha1(XII)$	fibril associated molecule

The biosynthesis of fully functional collagen molecules requires the concerted expression of collagen structural genes and genes coding for posttranslational modification enzymes. The primary products of translation of collagen mRNA molecules are polypeptides, prepro-α chains, that contain hydrophobic signal sequences found in most other secretory polypeptides. During or after ribosomal synthesis of the prepro-α chains, the chains are extensively modified by co-translational and posttranslational modifying enzymes. These modification reactions include conversion of preproα chains to proα chains by removal of signal sequences, as well as hydroxylation, glycosylation and disulfide bond formation. In the rough endoplasmic reticulum or in the Golgi region, the hydroxylated and glucosylated pro-α chains fold into a characteristic triple helix. Following secretion of procollagen from







1. Synthesis and entry of chain into lumen of rough ER, 2. Cleavage of signal peptide, 3. Hydroxylation of selected prolyl and lysyl residues, 4. Addition of N-linked oligosaccharides, 5. Glycosylation of hydroxylysyl residues, 6. Chain alignment formation of disulfide bonds, 7. Formation of triple helix procollagen, 8. Transport vescicle, 9. Exocytosis, 10. Removal of N- and C-terminal pro sequences, 11. Lateral covalent cross-linking of tropocollagens, 12. Aggregation of fibrils.

FIGURE 1: Major events in the biosynthesis of collagen. Covalent modifications of the collagen polypeptide include hydroxylation, glycosylation, disulfide bond formation and proteolytic cleavages; these occur in a precisely defined sequence in the rough ER, Golgi vesicles and the extracellular space. The modifications allow formation of a stable triple-stranded collagen helix and also the lateral alignment and covalent cross-linking of collagen helixes into 50-nm-diameter collagen fibrils. These in turn aggregate to form the collagen fibers. From: Molecular Cell Biology, by Darnell, Lodish, Baltimore 1986. (with permission)

cells, the procollagen molecule is converted to collagen by the action of specific proteolytic enzymes (figure 1) [for review see 31,32].

The collagen molecule consists of three polypeptide chains, each containing 1050 amino acids. The amino acid sequence of collagen is extremely regular: every third amino acid in each chain is glycine. This feature is essential for the chains to fold into the triple helix. Proline is also abundant and the repeating sequences glycine-proline-X and glycine-proline-hydroxyproline occurs very frequently.

REGULATION OF COLLAGEN METABOLISM:

The changing patterns of the connective tissue during growth, development and repair following injury, require a delicate balance between synthesis and degradation of collagen [12]. The interstitial collagens are degraded, predominantly extracellularly, by specific collagenases (metalloproteinases) capable of cleaving within the central helical region across the three chains at a single locus, which reduces the thermostability of the collagen fibril. Collagenolysis is modulated at the level of cellular production of latent collagenase, activation of latent collagenase and the production of collagenase inhibitors [33].

The extracellular matrix is composed of a large variety of multi-domain molecules. Initially it was assumed that these represent the structurally stable material surrounding fibroblasts; however, there is growing evidence that the extracellular matrix not only acts as the structural scaffold of the tissue but can also provide signals for cells, thus controlling several cellular functions [34,35]. For example, the growth of fibroblasts is reduced when cells are embedded in a three-dimensional collagen matrix [36] and it has also been shown that contact of fibroblasts with mature collagen I fibrils leads to a reduction of collagen synthesis and to activation of collagenase gene expression [37].

Over the recent years it has become increasingly clear that cellular mediators like cytokines and growth factors are able to stimulate the course of matrix deposition during healing and thus accelerate wound repair [38]. In this respect positive effects have been reported for interleukin-1 (IL-1), interleukin-2 (IL-2), transforming growth factor beta (TGF-ß), fibroblast growth factor (FGF), epidermal growth factor (EGF),

insulin-like growth factor (IGF) and platelet derived growth factor (PDGF) [39-44]. EGF has been used with encouraging results in a clinical trial [45,46]. Such studies start from the assumption that these proteins are intrinsically involved in the repair process and that one or several of them may be critical to the healing of wounds. Indeed, it has been shown that cytokines and growth factors are expressed within the wound area. Wound macrophages express mRNA for TGF-α, TGF-ß, PDGF and IGF [47] and an increased expression and accumulation of TGF-ß, PDGF and FGF has been found at wound sites [48-51]. In addition, antibodies against FGF are able to retard wound healing [52]. The participation of cytokines IL-1 and TNF in the early inflammatory response to wounding may be derived from their appearance, both on mRNA and protein level, in wound chambers [53].

Most information concerning the regulation of collagen metabolism by cytokines has been obtained from in vitro studies and little is known about their role in vivo. Cytokines are released by a variety of cell types during various stages of wound healing where they probably act together in a complex concert and the actual metabolic control of tissue events depends on the relative concentration of individual cytokines. It has been reported that IL-1 stimulates collagen synthesis in fibroblasts at a pretranslational level [54]. However, we (cf. chapter 6) and other investigators have shown that IL-1 is also capable of reducing collagen synthesis in fibroblasts from dermal tissue [55,56]. Decreased synthesis of collagen type I and III is also seen after incubation of fibroblasts with gamma interferon. This effect is accompanied by diminished steady state levels of the corresponding mRNA due to decreased gene transcription [57]. TNFα also reduces collagen synthesis, induces collagenase gene expression and acts synergistically with IFN-gamma in reducing collagen synthesis [58]. Thus, combined activity of different cytokines might be responsible for the effective up and down regulation of collagen gene expression, in association with collagen synthesis, throughout the different stages of wound healing.

As mentioned above, it is well known that many growth factors such as TGF-ß, PDGF and EGF promote the wound healing process in vivo [49,59-61]. These effects are thought to be the result of stimulation of cell migration and proliferation and synthesis of extracellular matrix proteins [62,63]. TGF-ß, in particular, is capable of stimulating collagen synthesis at the pretranslational level [62].

COLLAGEN IN THE INTESTINAL TRACT:

The wall of the intestine is made of four concentric layers of different tissues: the serosa, the muscularis, the submucosa and the mucosa (figure 2) [64]. The mucosa is the most important layer with respect to the digestive and absorptive function of the intestine. The submucosa forms the backbone of the intestinal wall. This layer mainly consists of collagen [65], which determines to a large extent the integrity of the intestinal wall and its capacity to withstand intraluminal pressure.

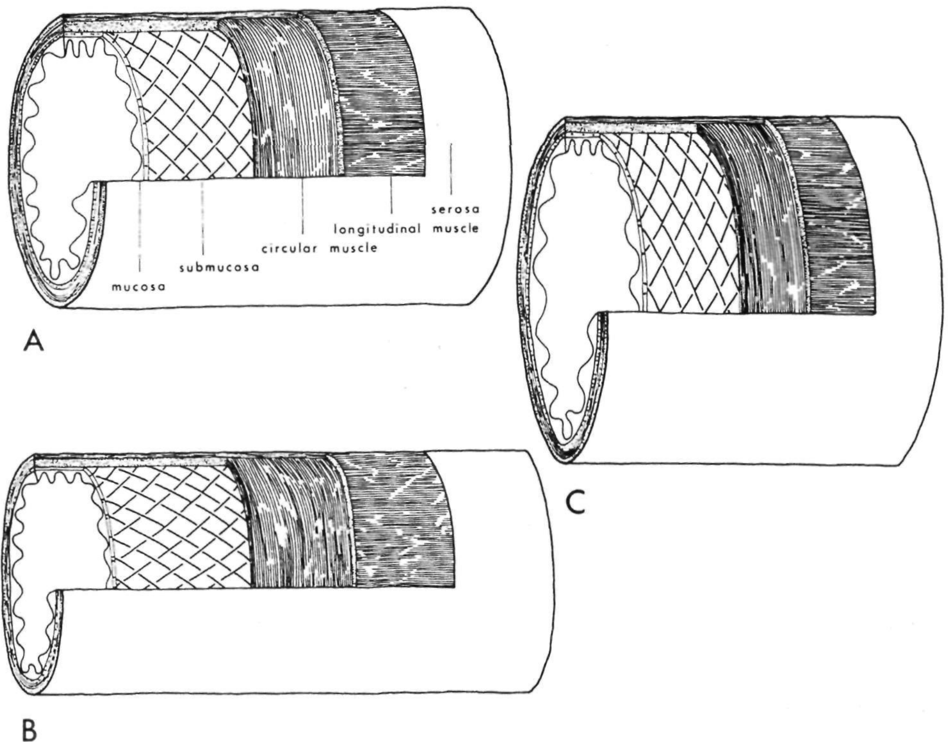


FIGURE 2: Schematic presentation of the wall of the small intestine, showing the orientation of the musculature in the circular and longitudinal muscle layers and that of the collagen fibers in the submucosa. In **A** the intestine is moderately distended along both axes (as when fluid is injected into the lumen). In **B** the intestine is distended mainly along its length. In **C** the intestine is mainly radially distended. From: Gabella G. The cross-ply arrangement of collagen fibres in the submucosa of the mammalian small intestine. Cell Tissue Res 1987; 248: 491-497. (with permission)

The major collagen type in human intestine is type I, but the presence of significant amounts of type III and type IV has also been reported [66]. The dense arrangement of collagen fibers in the submucosa can be shown clearly by both transmission and scanning electron microscopy [67].

The main framework of the submucosa is composed of two arrays of collagen fibers running diagonally around the intestinal wall, one set as a clockwise helix and the other as a counter-clockwise helix. These two sets of helices, showing mirror-image symmetry are oriented at a ± 35-50° angle to the longitudinal axis of the intestine (figure 3) [68]. When the collagen-fibre lattice makes an angle of 50-55° with the longitudinal axis of the intestine, the lumen has reached its maximum volume [69].

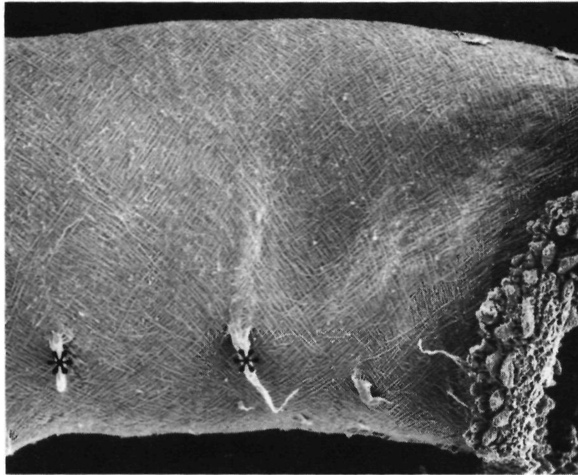


FIGURE 3: A scanning electron micrograph showing the collagenous frame work of the submucosa of the rat small intestine. Collagen fibers are arranged in two sets of helices that interweave. Angles formed by two helices are slightly different, depending on the area of the wall. The everted mucosa with villi is seen in the top right hand corner. From: Kumora T. The lattice arrangement of the rat small intestine: scanning electron microscopy. Cell Tissue Res 1988: 251: 117-121. (with permission)

The collagen fibers generally measure about 5 μm in diameter and each collagen fiber consists of bundles of collagen fibrils about 80 nm in diameter. This diagonal arrangement of collagen fibers has been well documented in different species

(including rat, dog, pig and human) by various methods [70-73]. The diagonal orientation of the fibers is essential for the flexibility of the submucosa in allowing the deformation of the intestine during peristalsis. Despite the non-elastic nature of collagen, the submucosa can adapt to the various shapes of the intestinal lumen by a simple change in the angles formed by the fibers (figure 2) [64,74]. Scanning electron microscopy has revealed that these collagen fibers of dual array do not constitute two separate layers, but interweave to form a unified sheet [68].

The strength of the intestinal anastomosis depends, in the initial phase of the wound healing sequence, on the suture holding capacity of the existing collagen fibers [75], while newly formed collagen fibrils bridge the anastomosis and restore the original, preoperative strength of the healing intestine. Thus, postoperative collagen synthesis and degradation are expected to affect anastomotic strength and the equilibrium between both processes is thought to be crucial to intestinal healing [12]. Cells that are capable of synthesizing collagen are endothelial cells [76], smooth muscle cells [77] and fibroblasts [78]. The latter are thought to be mainly responsible for collagen synthesis in the wound area.

MEASUREMENT OF COLLAGEN SYNTHESIS:

The anastomotic collagen content is the net result of collagen degradation and collagen synthesis and does not allow any quantitative conclusions concerning the individual processes. An elevated collagen content may be caused by limited degradation or an increased synthesis or, for that matter, by an increased degradation coupled with an even more strongly increased synthesis. In order to draw conclusions on the course of postoperative collagen synthesis, the latter process should be measured independently.

Methods to measure collagen synthesis exploit the fact that hydroxyproline is almost uniquely found in collagen, with only small amounts occurring in elastin and nonconnective tissue proteins, such as the C1q component of complement [79] and the enzyme acetylcholine esterase [80]. Radioactive proline is used as a precursor to label newly synthesized collagen molecules and other non-collagenous proteins. Tissue or cell homogenates are dialyzed extensively or the proteins in them are precipitated

with trichloroacetic acid in order to remove most of the nonincorporated radioactive proline. The labeled proteins are then hydrolysed and subsequently the hydroxyproline may be separated from the proline by ion exchange chromatography on a Dowex-50 column [81], thin layer chromatography [82], high performance liquid chromatography (HPLC) [83] or by a technique using chloramine-T oxidation and toluene extraction of pyrrole which is formed from the oxidized product of hydroxyproline after heating [84].

Another way to measure collagen synthesis, is to treat [^{3}H]proline labeled tissue or cells with bacterial collagenase as described by Peterkofsky et al. [85]. The use of bacterial collagenase allows the measurement of collagen polypeptide synthesis independent of the process of proline hydroxylation. A most important aspect of this technique is that the collagenase used should be free of any nonspecific proteolytic activity [86]. This method to measure collagen synthesis is, and has been, used in a multiplicity of studies on in vitro collagen synthesis in different types of cells and tissue explants [56,87-89].

COLLAGEN SYNTHESIS IN EXPERIMENTAL INTESTINAL ANASTOMOSES:

Anastomotic hydroxyproline concentrations change massively during the first postoperative days. This phenomenon was first reported by Cronin et al. [90], who found a transiently lowered hydroxyproline concentration in colonic anastomoses of the rat. The finding was confirmed by other authors and similar results were obtained in other species [91-93]. The postoperative hydroxyproline concentration in ileum anastomoses is also reduced but to a lesser degree than in colon [93-95]. Changes in hydroxyproline and, by extrapolation, collagen concentrations do not necessarily represent absolute changes in the amount of collagen present in the bowel wall. In this respect the hydroxyproline present per unit intestinal length, is a better measure [96]. This way, an increase in anastomotic hydroxyproline content has been found within three to four days after operation, both in ileum and colon [97,98], which must be the result of de novo collagen synthesis.

In vivo collagen synthesis in intestinal anastomoses can be quantitated after injection of radioactively labeled proline at a fixed time before sacrifice. Subsequently,

the incorporation of label into hydroxyproline is determined and its specific (dpm labeled hydroxyproline per mole hydroxyproline per biopsy) activity is a measure for the amount of newly synthesized collagen. Utilizing this method, the occurrence of increased post-operative collagen synthesis in the anastomotic area was first demonstrated by Cronin et al. [99] and confirmed by Jiborn et al. [100]. From two days after operation onwards, collagen synthesis in and around colonic anastomoses was increased compared to the collagen synthesis measured in the colon of unoperated control animals [101]. The same phenomenon occurs around anastomoses in the small intestine [102]. The course of anastomotic collagen synthesis may thus be followed and compared under various conditions thought to affect anastomotic repair. So far, application of this technique in the research on intestinal healing has been limited, possibly because it requires administration of large amounts of (expensive) labeled proline to living animals.

The establishment of an in vitro system to measure collagen synthesis in uninjured and anastomotic intestinal tissue would have the advantages that, compared to the in vivo system, only a fraction of the relatively expensive radioactive proline is required. Secondly, no special animal care is required because the animals are not injected with a radioactive compound. Finally, with the in vitro system, it should be possible to measure collagen synthesis in the anastomotic area shortly after operation, whereas with the in vivo system this is only possible 24 hours after operation, because the precursor needs to be injected at least 24 hours prior to sacrifiction.

AIM OF THE STUDY:

Anastomotic repair in the large intestine is disturbed rather frequently. This appears to be in contrast to healing in the small bowel. The formation of new collagen fibrils is essential for the development of anastomotic strength. So far, relatively little is known about the characteristics of collagen synthesis in both bowel segments under various conditions and about its regulation. The experiments described in this thesis have been devised to further characterize this process which is most crucial to anastomotic healing.

The aims of the study were

- to develop an in vitro system to measure collagen synthesis in intestinal tissue;
- to quantitate collagen synthesis throughout the intestinal wall (from duodenum to sigmoid);
- to characterize the post-operative changes in collagen synthesis which occur in anastomoses in ileum and colon;
- to characterize the effects of in vivo administration of antineoplastic agents on anastomotic collagen synthesis;
- to study the effects of by antineoplastic agents and various regulatory compounds on collagen synthesis in fibroblasts from skin and colon

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CHAPTER 2

COLLAGEN SYNTHESIS IN INTESTINAL EXPLANTS IN THE RAT

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SUMMARY

Collagen is the major structural component of the intestinal wall and its metabolism is of special interest for intestinal strenght. We describe collagen synthesis in short time (3h) incubations of rat intestinal tissue, as measured in terms of incorporation of [^{3}H]proline in collagenase-digestible protein and percentage relative collagen synthesis. In this time span, incorporation of [^{3}H]proline in collagen increases linearly with time and tissue weight. Addition of unlabeled proline during incubation, in excess of the 0.1 μM [^{3}H]proline always present, strongly increases both total protein and collagen synthesis suggesting that proline transport is rate limiting. Further experiments have been performed in the presence of labeled proline alone and with the addition of 3.5 mM unlabeled proline. Collagen synthesis is significantly higher in colon than in ileum, comprising 0.37% and 0.21%, respectively, of total protein synthesis. Also, collagen synthesis decreases considerably with age, both in ileum and colon.

The results presented here demonstrate that rat intestinal explants synthesize measurable amounts of collagen in vitro and that the system used is able to detect changes in in vivo synthetic capability such as induced by ageing.

INTRODUCTION

The connective tissue layer of the intestine, the submucosa, forms the backbone of the intestinal wall. This layer consits mainly of collagen [1], which protein thus determines, to a large extent, the integrity of the intestinal wall and its capacity to withstand intraluminal pressure. As a consequence, collagen metabolism and thus the factors regulating collagen degradation and synthesis are of particular interest for maintaining intestinal strenght.

This is the more so after intestinal resection and anastomosis.Anastomotic dehiscence is a major complication of surgery of the intestine, occuring more frequently in the large bowel (colon) than in the small bowel (ileum) [2,3].Post-operative collagen concentrations change massively around experimental colonic anastomoses [4,5] and, to a lesser extent, around ileal anastomoses [6,7]. This phenomenon is thought to reflect the successive occurence of collagen degradation and collagen synthesis. The equilibrium between these two processes is of critical

importance for normal healing [8]. Thus, examination of collagen synthesis and its regulation are essential in investigations aiming to improve healing of intestinal anastomoses or, indeed, of any wound [9].

A method has been described [10], and used extensively by the authors, to measure in vivo collagen synthesis around intestinal anastomoses. However, such a system presents certain limitations, both in terms of costs and its suitability to study regulation. Therefore, our aim has been to measure collagen synthesis in vitro in intestinal explants. While numerous publications describe in vitro collagen synthesis in isolated cells, particularly in fibroblasts, experience with tissue explants is limited. Also, in most in vitro systems collagen synthesis is quantitated, by measuring the incorporation of labeled proline into collagen, in the presence of labeled proline without the addition of unlabeled proline. Under these conditions proline concentrations remain far below those which may be regarded as physiologcal [11]. Therefore we have compared collagen synthesis in the presence of both high and low proline concentrations.

The present communication shows that explants of rat intestine produce measurable amounts of collagen, compares synthesis in ileum and colon, and examines the effect of age of the donor animal.

MATERIALS AND METHODS

animals:

Male wistar rats with an approximal weight of 225 gram (unless indicated otherwise) were used. They were fed a standard diet (Hope Farms, Woerden, The Netherlands) an allowed water ad libitum. The animals were sacrificed by an intraperitoneal overdose of sodium pentobarbital and the intestine was collected immediately.

materials:

l-[2,3-^{3}H]Proline (300 mCi/mg) and [^{3}H]Tryptophan (146 mCi/mg) were purchased from Amersham International, England. Dulbecco's Modified Eagles Medium (DMEM) was obtained from Gibco, Breda, The Netherlands. Collagenase (type 7) , deoxyribonucleic acid (calf thymus) , proline and bovine serum albumin (BSA) were all obtained from Sigma, St-Louis, USA. The scintilation liquid used was

picofluor-30 from Packard,Groningen, The Netherlands. Kanamycine was obtained from Gist-Brocades,Delft, The Netherlands. All other reagents were of analytical grade (Merck, Darmstad, Germany).

labeling of explants:

Samples of colon or ileum explants were cut into pieces of approximately 1-2 mm^2 and 75 mg (wet weight) tissue was transferred to a petri dish (diameter 35 mm). The pieces were washed once with physiological salt solution an once with incubation medium (DMEM containing 50 μg/ml ascorbate and 250 μg/ml kanamycine). Subsequently 1.5 ml incubation medium was added and the samples were incubated for 30 min at 37°C (95% air; 5% CO_2). The medium was then removed by suction and replaced with 1.5 ml incubation medium with 4.5 μCi [2,3-^{3}H]proline with or without the addition of 0.35 mM unlabeled proline. Incubation proceeded for 3hr. All subsequent steps were carried out at 4°C. The explants and medium were transferred to a centrifugation tube and spun for 5 min at 2500x g. The sediment was homogenized in 3.0 ml 50 mM Tris-HCl, pH 7.6, containing 25 mM EDTA, 10 mM NEM, 1 mM PMSF and 1 mM proline. TCA was added (final concentration 0.6M) to the homogenate which was then centrifuged for 5 min at 2500x g. The sediment was washed three times with 0.3M TCA containing 1 mM proline.

The above procedure describes the conditions ultimately chosen as standard method. In order to determine these conditions experiments were carried out, described in the text, where variations were used for incubation time, weight of the samples and concentrations of unlabeled proline.

collagenase treatment:

The final sediment was dissolved in 0.75 ml 0.2 N NaOH and neutralised by the addition of 0.3 ml 1 M HEPES and 0.3 ml 0.15 N HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to determine proline incorporation into collagen [12] 0.2 ml 20 mM TrisHCl, pH7.6, containing 50 mM $CaCl_2$ and 0.1 ml collagenase (chromatographically purified on a G200 gelfiltration column) were added to a 0.5 ml aliquot of the solubilized sample and the mixture was incubated for 5 hr at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.6M and 3 mM, respectively. After centrifugation (10 min; 14.500x g) a 1.0 ml aliquot of the

supernatant was counted in a liquid scintilation analyzer. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase yielded the collagen specific incorporation, which will be referred to as collagenase digestible protein (CDP). Substraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non collagenous protein (NCP).

The relative collagen synthesis was calculated with the formula [12] that takes into account the enrichment of proline in collagen compared to other proteins :

$$\% \text{ relative collagen synthesis} = \frac{CDP}{(NCP \times 5.4) + CDP} \times 100\%$$

The specificity of this method was tested by labeling intestinal explants with 4.5 μCi [^{3}H]tryptophan according to the procedure described above and measuring the radioactivity released by subsequent collagenase treatment.

other procedures:

DNA and protein were measured in the NaOH-solubilized TCA sediment, DNA by the method of Burton [13] using calf thymus DNA as a standard and protein by the method of Lowry et al [14] using bovine serum albumin as a standard.

RESULTS

Incubation of finely minced intestinal tissue with [^{3}H]proline resulted in incorporation of the label into protein in a time dependent fashion. The course of incorporation is shown in figure 1, where NCP is virtually equivalent to total protein. Treatment of the NaOH-solubilized protein fraction with purified collagenase released radioactivity (figure 2), presumably by degradation of collagen. Up until 4h additional radioactivity was released. At that time, addition of a second volume of collagenase had no effect. Also, doubling the amount of collagenase from the start did not result in an increased digestion rate of collagen (results not shown). For this reason an incubation time of 5h was chosen as a standard.

In order to exclude the possibility that the purified collagenase degraded other proteins than collagen, ileal explants were incubated with [^{3}H]tryptophan and subsequently

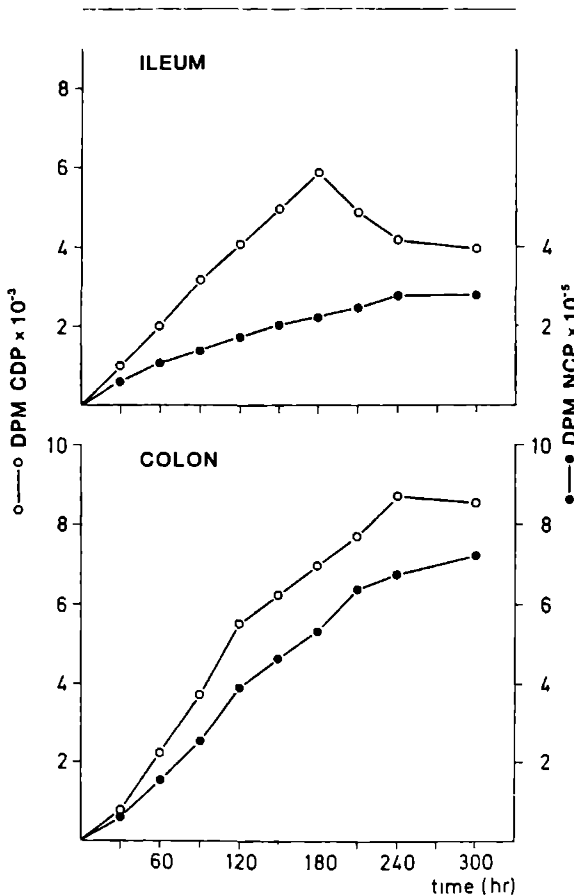

Figure 1: Synthesis of collagenase digestible protein (CDP, o) and non collagenous protein (NCP, ■) in intestinal explants as a function of time. Explants consisted of 75 mg tissue and were incubated in 0.1 μM radioactive proline.

treated with collagenase. Collagen does not contain tryptophan and therefore collagenase-mediated release of radioactivity from tryptophan labeled protein would mean that the enzyme contained proteolytic activity other than collagenase. However, while incorporation of [^{3}H]tryptophan in total protein, approximately 2000 dpm/mg wet weight, was in the same order of magnitude as that of [^{3}H]proline, the collagenase incubation failed to release a significant number of counts.

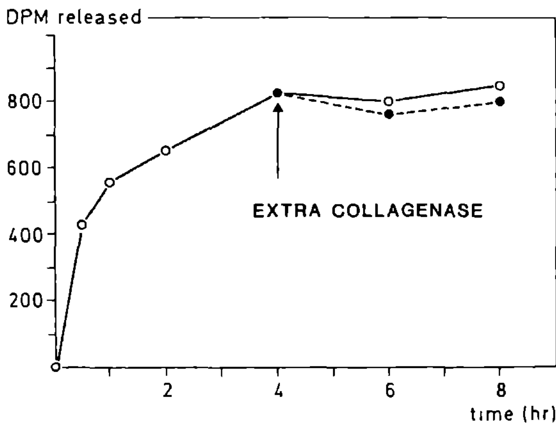

Figure 2: Collagenase mediated release of proline from ileal explants. A batch of ileal material was incubated for 3h with 0.1 μM radioactive proline and labeled protein was isolated by TCA precipitation.Samples of the sediment, equivalent to 100 mg explant material, were digested with increasing duration with 0.1 ml purified collagenase and the radioactivity released was counted (○). An additional 0.1 ml collagenase was added to a number of samples after 4h and incubation proceeded for another 2 or 4 h (●).

All data given here on the incorporation of [^{3}H]proline into protein in short-time tissue explants concern the tissue itself: the incubation medium was always discarded after centrifigation because it did contain only negligible amounts of CDP. The tissue showed significant incorporation into CDP which increased linearly with time over the first hours of incubation (figure 1). However, it appeared that after three hours in ileum and four hours in colon, no additional CDP was formed and that the size of this fraction was even diminished. This phenomenon was observed in several experiments and therefore the incubation time was routinely set at three hours.

Both the NCP and CDP fractions increased with the amount of tissue present (figure 3). If the tissue wet weight exceeded 75 mg incorporation leveled off, especially in ileum. The relative collagen synthesis remained practically unchanged over the weight range investigated. In further experiments 75 mg tissue was used, both for ileum and colon.

Measurement of collagen synthesis in vitro, either in cell or in tissue cultures, is usually performed in the presence of radiolabeled proline only : no unlabeled proline is added. Therefore, the proline concentration remains very low , in our experiments approximately 0.1 μM. We did examine the effect of addition of unlabeled proline to the incubation medium (figure 4). Addition of unlabeled proline did not result in a proportional decrease of radioactivity incorporated into either CDP or NCP. For instance, in ileum a molar excess of 10^3 only decreased the radioactivity in both NCP

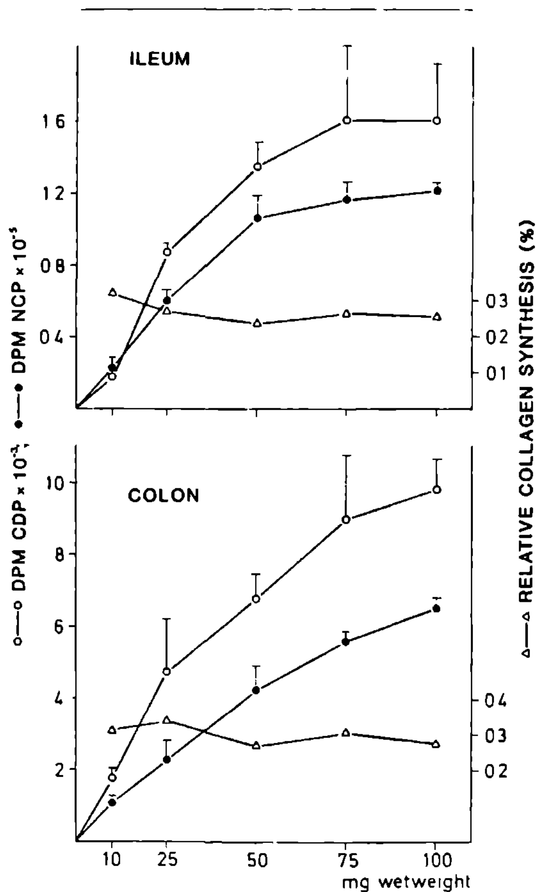

Figure 3: Synthesis of collagenase digestible protein (CDP,o) and non-collagenous protein (NCP, ●) as a function of the amount of tissue incubated. Triplicate samples of intestinal material were incubated for 3h in the presence of 0.1 μM radioactive proline. Values are given as average (± SD).

and CDP by 18%. For colon, this percentage was 26 for NCP and 54 for CDP. At a labeled proline/unlabeled proline ratio of 1:10^5 a measurable incorporation of radioactivity was still observed, even in the CDP fraction. Our interpretation of these results (see discussion) is that collagen synthesis, as reflected by the radioactivity in CDP, proceeds far below the synthetic capacity in the presence of [^{3}H]proline alone. Addition of unlabeled proline strongly increases the synthesis rate. Therefore, we

measured the synthesis in further experiments under two conditions: in the presence of [^{3}H]proline alone, where the proline concentration is approximately 0.1 μM, and in the presence of added unlabeled proline in a final concentration of 0.35 mM.

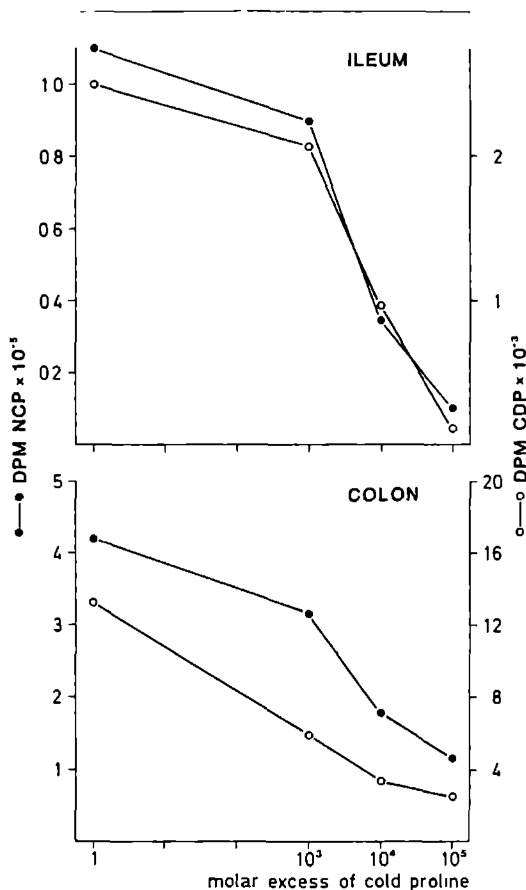

Figure 4: Synthesis of collagenase digestible protein (CDP, o) and non-collagenous protein (NCP, ●) as a function of proline present at incubation. Intestinal explants (75 mg) were incubated for 3 h with 0.1 μM radioactive proline in the absence (represented on the horizontal axis by the 1 molar excess point) or presence of a large excess of unlabeled proline.

With these standard conditions incorporation of [^{3}H]proline in CDP was measured in ileum and colon of six rats. Significant differences were found between ileum and colon, both in the absence (table 1) and in the presence (table 2) of

unlabeled proline during the incubation. Incorporation in CDP was twice as high in colon as in ileum. This difference was always present, regardless if incorporation was expressed on the basis of wet weight, protein or DNA. Also, the relative collagen synthesis was significantly higher in colon. These differences were found both at low (absence of unlabeled proline) and high (presence of 0.3mM unlabeled proline) proline concentrations, although they seemed somewhat more pronounced under the former condition. The fact that these values indeed represent newly formed protein was confirmed by incubation of the explants in the presence of 0.1 mM cycloheximide, which inhibited incorporation in both NCP and CDP by more than 90%.

Table 1: Incorporation of [^{3}H]proline in collagenase-d gestible (CDP) and non-collagen protein (NCP). Intestinal explants were incubated under standard conditions (75 mg, 3h) in the presence of 4.5 μCi [^{3}H]proline. Data represent the average values (±SD) from six separate experiments. Differences between ileum and colon were tested for significance using a signed rank test.

	ILEUM	COLON	COLON/ILEUM	p-value
NCP:				
-dpm/mg ww	2369 ± 360	5275 ± 1260	2.3 ± 0.8	0.03
CDP:				
-dpm/mg ww	48 ± 6	141 ± 39	3.1 ± 1.1	0.03
-dpm/mg protein	401 ± 119	904 ± 216	2.4 ± 0.8	0.03
-dpm/μg DNA	28 ± 5	58 ± 17	2.1 ± 0.7	n.s.
% relative collagen synthesis	0.21 ± 0.03	0.37 ± 0.07	1.8 ± 0.5	0.03

(n.s.= not significant)

Table 2: Incorporation of [^{3}H]proline in collagenase digestible (CDP) and non-collagenous protein (NCP) in the presence of an excess of unlabeled proline. Intestinal explants were incubated under standard conditions (75 mg, 3h) in the presence of 4.5 μCi [^{3}H]proline plus 0.35 mM unlabeled proline. Data represent the average values (± SD) from six separate experiments. Differences between ileum and colon were tested for significance using a signed rank test.

	ILEUM	COLON	COLON/ILEUM	p-value
NCP				
-dpm/mg ww	1328 ± 274	1985 ± 243	1.6 ± 0.4	0.03
CDP				
-dpm/mg ww	30 ± 6	56 ± 13	1.9 ± 0.7	0.03
-dpm/mg protein	249 ± 87	353 ± 91	1.6 ± 0.7	n.s.
-dpm/μg DNA	16 ± 5	25 ± 8	1.7 ± 0.8	n.s.
% relative collagen synthesis	0.24 ± 0.02	0.39 ± 0.09	1.7 ± 0.4	0.03

(n.s.= not significant)

Finally, we investigated the effect of age of the donor animals on collagen synthesis. The values in young adult animals (± 7 weeks old, approximate weight 225 gram) normally used in our experiments were compared to those obtained from young

(3 weeks,approximate weight 50 gram) and adult (13 months, approximate weight 575 gram) rats. Clearly, incorporation in CDP depended strongly on age, both in ileum (table 3) and colon (table 4). In these tables, incorporation in CDP is expressed on the basis of protein but similar results were obtained if data were calculated using wet weight or DNA. All values obtained in young adults were compared, by means of a Wilcoxon 2-sample test, to those measured in young and adult animals, respectively. In all cases the differences observed with this limited number of animals were significant ($p < 0.05$), collagen synthesis being higher in young rats and lower in adult rats if compared to young adult animals. This picture was independent of the proline concentration. The age effect, particularly the decrease in incorporation from young adults to adults, appeared somewhat more pronounced in colon than in ileum.

Table 3: Effect of age on incorporation of [^{3}H]proline in collagenase digestible protein (CDP) in ileal explants. Ileal tissue was incubated under standard conditions (75 mg, 3h) with [^{3}H]proline in the presence or absence of 0.35 mM unlabeled proline. Data are given as average value ± SD.

	YOUNG (n=3)	YOUNG-ADULTS (n=6)	ADULTS (n=3)
- unlabeled proline			
CDP dpm/mg protein	1018 ± 142	401 ± 119	115 ± 30
%relative collagen synthesis	0.36 ± 0.04	0.21 ± 0.03	0.15 ± 0.01
+ unlabeled proline			
CDP dpm/mg protein	618 ± 41	249 ± 87	84 ± 35
%relative collagen synthesis	0.48 ± 0.06	0.24 ± 0.02	0.20 ± 0.06

Table 4: Effect of age on incorporation of [^{3}H]proline in collagenase-digestible protein (CDP) in colonic explants. Colonic tissue was incubated under standard conditions (75 mg, 3h) with [^{3}H]proline in the presence or absence of 0.35 mM unlabeled proline. Data are given as average value ± SD.

	YOUNG (n=3)	YOUNG/ADULTS (n=6)	ADULTS (n=3)
- unlabeled proline			
CDP dpm/mg protein	2480 ± 536	904 ± 216	336 ± 127
% relative collagen synthesis	0.69 ± 0.06	0.37 ± 0.07	0.21 ± 0.01
+ unlabeled proline			
CDP dpm/mg protein	968 ± 245	353 ± 91	93 ± 17
% relative collagen syntheiss	0.74 ± 0.05	0.39 ± 0.09	0.21 ± 0.01

DISCUSSION

Collagen synthesis is an essential feature of wound repair. We have studied the post-operative changes in collagen concentrations around intestinal anastomoses [6,7], which are affected by e.g. infection [15] and chemotherapy [16]. In order to asses the role of a changed rate of collagen synthesis, under conditions which influence anastomotic healing, a system should be available which allows quantification of this process. So far, no attempts have been described to measure collagen synthesis in tissue explants of intestine. A tissue culture system has been used for the study of wound healing in skin [17]. The relative collagen synthesis in rat skin biopsies was found to average 0.61%. However, skin contains far more collagen (up to 70% of tissue dry weight) than intestine, where this protein comprises less than 10% of dry weight [18]. Also, collagen turnover in intestine has been reported to be far lower than in skin [19]. Still, the present results, which show a significant incorporation of [^{3}H]proline in CDP, and a relative collagen synthesis approximating that in skin, in short-time explants of both ileum and colon, indicate the presence of considerable collagen synthetic potential in uninjured intestine.

The method used, incorporation of [^{3}H]proline in protein and quantification of the newly-formed collagen by measurment of the collagenase digestible fraction, is firmly established and widely used in research on collagen synthesis [12]. The premises that the radioactivity in CDP is indeed a specific measure for the collagen formed in intestinal explants is confirmed by the fact that tryptophan labeled protein remains unaffected by the collagenase digestion, because tryptophan is absent from collagen. In most systems the time used for the collagenase treatment is 1.5 h [16]. In our system, an incubation time of 1.5 h results in sub-maximal release of radioactivity (figure 2) and thus underestimation of collagen synthesis. Digestion appears to be complete only after at least 4 h. The digestion rate is not limited by the amount of enzyme present since doubling the collagenase concentration does not affect the reaction. Probably, penetration of the enzyme is time-consuming and rate-limiting.

Incorporation of label in CDP increases linearly with time for at least 3 h. Thereafter, the amount of [^{3}H]proline in CDP decreases, particularly in ileum (figure 1), indicating massive degradation of newly synthesized collagen. It is known that intracellular degradation of newly synthesized collagen occurs: labeled hydroxyproline

is detected in small molecular weight fragments within minutes after in vitro labeling with proline [20]. Recently, the extent of this process in various rat tissues has been demonstrated in vivo [21]. Therefore, it seems likely that degradation of newly synthesized collagen occurs all along the incubation period. Apparently, after a number of hours synthesis decreases or degradation increases to such an extent that the net result is a diminished incorporation in CDP. For this reason, the incubation time has been limited to 3 h.

In vitro collagen synthesis is usually measured in the absence of unlabeled proline. The effect of changing proline concentrations has been studied in cell cultures [22] but has, to our knowledge, not been reported for tissue cultures. In the present system, collagen synthesis appears to be strongly influenced by addition of unlabeled proline. Incorporation of [^{3}H]proline in CDP falls by only 18% in ileum and 54% in colon when a 1000-fold molar excess of unlabeled proline is added (figure 4). If the synthesis would have remained at the same level, incorporation of [^{3}H]proline would only be 0.1 % of that in the presence of [^{3}H]proline alone, assuming an even distribution and equal chances for incorporation into protein for labeled and unlabeled proline. Thus, under these conditions synthesis increases enormously, by a factor 820 in ileum and 460 in colon. This effect is even more pronounced at higher dilutions with unlabeled proline. An explanation of these results might be found in the fact that the rate of collagen synthesis depends strongly on the rate of transport of proline across membranes. The Km for proline, like those of other amino acids, is fairly high and approximates 6.5 mM [23]. Thus, if only [^{3}H]proline is present and the overall proline concentration is only 0.1 μM, transport presumably is the rate limiting factor, resulting in collagen synthesis far below the capacity of the system. For this reason we have investigated collagen synthesis under two conditions: in the presence of labeled proline alone and in the presence of 0.35 mM unlabeled proline. This latter concentration is close to that found in rat plasma [11] and at the same time yields measurable incorporation of [^{3}H]proline in CDP. It should be emphasized that differences found, between ileum and colon and between rats of various ages, are always similar, whether measured in the absence or presence of unlabeled proline.

Collagen synthesis is higher in colon and ileum. This effect is observed persistently and not only the result of a higher overall protein synthesis because the

relative collagen synthesis is also significantly higher in colon. The percentage relative collagen synthesis in intestinal explants, which averages 0.2% in ileum and 0.4% in colon, approximates the 0.6% reported for tissue cultures of skin [17] and lung [24].

Collagen synthesis in skin depends on age [25]. Early work with tissue biopsies has shown the collagen synthesis to decrease with age of the donor [26]. We have found a similar effect in intestinal explants where synthesis is significantly reduced in older animals, both in terms of absolute [^{3}H]proline incorporation in CDP and in terms of relative collagen synthesis. These results also support the notion that the current system, using short time intestinal explants, is capable of reflecting changes which have taken place in the living species and therefore is a valuable tool to study collagen synthesis in the intestine in health and disease.

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CHAPTER 3

POSTOPERATIVE CHANGES IN COLLAGEN SYNTHESIS IN INTESTINAL ANASTOMOSES OF THE RAT.

Differences between small and large bowel.

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ABSTRACT

Collagen synthesis is an essential feature of anastomotic healing in the intestine. We have studied postoperative collagen synthesis, measured in vitro, in intestinal anastomoses from 3 hours to 28 days after operation. For this purpose, both an ileal and a colonic anastomosis were constructed within the same animal and the results in both intestinal segments were compared.

In ileum, collagen synthesis was already significantly increased, with respect to unoperated controls, 3 hours after operation. It remained elevated during the period of study, with a maximal 10-fold stimulation 4 days after operation, and had nearly returned to the preoperative level after 4 weeks. The general pattern was the same in colon, although quantitatively different: the increase in synthetic activity appeared delayed in comparison to ileum. Maximal stimulation was approximately 6-fold. In addition, we calculated for each rat the ratio between anastomotic collagen synthesis and the average value found in non-operated control animals. Postoperative stimulation in ileum was higher than in colon in almost every animal examined.

The results demonstrate that the ileum responds more quickly and strongly to wounding than the colon, at least as far as the production of new collagen is concerned. Possibly, this phenomenon contributes to the lower failure rate apparent for anastomoses in the small bowel.

INTRODUCTION

Dehiscence of intestinal anastomoses remains a major complication in surgical practice. Despite improved surgical techniques leakage of colonic anastomoses occurs rather frequently, resulting in high morbidity and mortality. In a much-cited study on operations for large-bowel cancer the overall incidence of leakage was 10.8 % in anastomoses constructed intraperitoneally [1].There is still much to be learned about the molecular processes which contribute to normal healing of the bowel wall: such research is essential in order to seek measures which could benefit healing and ultimately prevent anastomotic leakage.

The strength of both the intact and the anastomosed bowel wall is derived from collagen fibrils, located predominantly in the submucosa [2,3], which connective layer forms the backbone of the intestine. During the first postoperative days

anastomotic strength is low and anastomotic collagen levels change massively [4]. It is generally assumed that the transiently lowered collagen concentrations, observed during normal healing, are the result of early, although limited, collagen degradation followed by considerable collagen synthesis. While early anastomotic strength depends on the suture-holding capacity of existing collagen fibrils, newly-formed collagen is needed to bridge the gap and restore the original strength of the bowel wall. Thus, the progress of collagen synthesis plays a central role in the healing sequence: disturbance of its regulation will affect anastomotic strength and might enhance chances for dehiscence.

The occurrence of collagen synthesis around experimental intestinal anastomoses has been demonstrated in vivo, both in ileum [5] and in colon [6] from two days after operation onwards.The methodology used for such experiments requires administration of large amounts of radioactive proline to animals and precludes the measurement of synthesis in the immediate postoperative period since the precursor needs to be injected at least 24 h before sacrifiction [6]. We have recently established a system to measure collagen synthesis in intestinal explants [7]. This in vitro system allows measurement immediately after operation and appears suitable to initiate research on the effects of potentially regulatory factors. Here, we report on the postoperative stimulation of the collagen synthesis, as measured in anastomotic explants collected from three hours after operation onwards. As results from previous experiments have suggested that collagen synthesis is initiated at an earlier stage in the small intestine than in the large intestine [8], we have constructed both an ileal and a colonic anastomosis in the same animal and compared the postoperative course of collagen synthesis in both intestinal segments.

MATERIALS AND METHODS

animals:

Male wistar rats with an approximate weight of 225 g were used. They were fed a standard diet (Hope Farms, Woerden, The Netherlands) and allowed water ad libitum. The animals were sacrificed by an intraperitoneal overdose of sodium pentobarbital and the intestinal biopsies were collected immediately.

materials:

l-[2,3-^{3}H]Proline (300 mCi/mg) was purchased from Amersham International, England. Dulbecco's Modified Eagles Medium (DMEM) was obtained from Gibco, Breda, The Netherlands. Collagenase (type 7) , deoxyribonucleic acid (calf thymus) and bovine serum albumin (BSA) were all obtained from Sigma, St-Louis, USA. The scintillation liquid used was picofluor-30 from Packard, Groningen, The Netherlands. Kanamycin was obtained from Gist-Brocades, Delft, The Netherlands. All other reagents were of analytical grade (Merck, Darmstad, Germany). Suture material used was ethilon 8x0 (Ethicon, Norderstedt, FRG).

operative techniques:

All animals received an anastomosis in both ileum and colon. Surgery was performed under semi-sterile conditions using a Zeiss operating microscope. The rats were anaesthetized by an intraperitoneal injection with sodium pentobarbital. The abdomen was opened through a midline incision of approximately 4 cm. The ileum was transsected at 15 cm proximal to the ileal-coecal junction and an end-to-end anastomosis was constructed using 8 single-layer inverting interrupted 8x0 Ethilon sutures. Subsequently, the descending colon was transsected 3 cm proximal to the peritoneal reflection and continuity was restored as described above. The abdomen was closed in two layers using silk for the fascia and staples for the skin.

After 3 and 12 hours and 1, 2, 3, 4, 7, 14 and 28 days the animals were sacrificed (six at each time point) and the anastomotic segments were resected, opened longitudinally and washed twice with physiological salt solution.

Control segments were obtained from non-operated rats of similar age and weight: uninjured intestine was taken from the same sites where experimental animals received their anastomoses.

assay of collagen synthesis:

Collagen synthesis was measured in tissue explants, according to a procedure validated before for rat intestinal tissue [7]. The anastomosis proper (± 2 mm left and right of the transsection line) was isolated and cut into pieces of approximately 1-2 mm^2. Two equal samples, 35-70 mg wet weight, were transferred to petri dishes (diameter 35 mm). The pieces were washed once with physiological salt solution and once with incubation medium (DMEM containing 50 μg/ml ascorbate and 250 μg/ml

kanamycin). Subsequently, 1.5 ml incubation medium was added and the samples were incubated for 30 min at 37°C (95% air; 5% CO_2). The medium was then removed by suction and replaced with 1.5 ml incubation medium containing 4.5 μCi [2,3-^{3}H]proline. Incubation proceeded for 3hr. All subsequent steps were carried out at 4°C. Both tissue and medium were transferred to a centrifugation tube and spun for 5 min at 2500 x g. The sediment was homogenized in 3.0 ml 50 mM Tris-HCl, pH 7.6, containing 25 mM ethylenediaminetetraacetic acid (EDTA), 10 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM proline. Trichloroacetic acid (TCA) was added (final concentration 0.6M) to the homogenate which was then centrifuged for 5 min at 2500 x g. The sediment was washed three times with 0.3M TCA containing 1 mM proline.

The final sediment was dissolved in 0.75 ml 0.2 M NaOH and neutralized by the addition of 0.3 ml 1 M N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid) (HEPES) and 0.3 ml 0.15 M HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to determine proline incorporation into collagen 0.2 ml 20 mM Tris-HCl, pH 7.6, containing 50 mM $CaCl_2$ and 0.1 ml collagenase (chromatographically purified on a G200 gelfiltration column) were added to a 0.5 ml aliquot of the solubilized sample and the mixture was incubated for 5 hr at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.6M and 3 mM, respectively. After centrifugation (10 min; 14.500 x g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyzer. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase yielded the collagen specific incorporation, which will be referred to as collagenase digestible protein (CDP). Subtraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non collagenous protein (NCP).

The relative collagen synthesis was calculated with the formula [9] that takes into account the enrichment of proline in collagen compared to other proteins :

$$\text{\% relative collagen synthesis} = \frac{CDP}{(NCP \times 5.4) + CDP} \times 100\%$$

other procedures:

DNA was measured in the NaOH-solubilized TCA sediment, by the method of Burton [10] using calf thymus DNA as a standard.

Statistical methods are mentioned with the results.

RESULTS

The intestinal wall synthesizes collagen. We reported in an earlier communication that collagen synthesis can be measured in short-term explants of, previously uninjured, intestinal tissue [7]. The same procedure was applied to explants from intestinal anastomoses. In a first series of experiments, increasing amounts of tissue from 4-days-old anastomoses, constructed both in ileum and colon, were incubated with [^{3}H]proline for various time periods. Incorporation into the CDP fraction increased linearly with both time and tissue weight (results not shown). The standard conditions chosen for subsequent experiments , 50 mg tissue incubated for 3 h, fall well within the linear part of the respective curves.

Collagen synthesis around intestinal anastomoses is strongly enhanced as compared to that in uninjured intestine. For practical reasons, it was impossible to measure synthesis in control segments removed during operation, which would have enabled comparison between anastomotic and control segments from the same animal. The fact that anastomotic construction not only affects collagen synthesis in the anastomotic area but also at locations further removed, particularly in the small intestine (Martens and Hendriks, manuscript in preparation), precluded the use of control segments removed at sacrifiction. Since the combination of anaesthesia and abdominal incision did not influence intestinal collagen synthesis during the first week after laparotomy (results not shown) we used non-operated control rats to establish normal values.

First, it was established that anastomotic construction in the large bowel did not significantly increase collagen synthesis in the small bowel and vice versa. For this purpose, rats were operated either in ileum or colon and collagen synthesis was measured after 4 days in the unoperated intestine at the site where normally the second anastomosis would have been constructed. Collagen synthesis in uninjured ileum in animals with a colon anastomosis was 30 ± 18 (average ± SD, n=6) dpm

CDP/μg DNA, compared to 41 ± 17 dpm CDP/μg DNA in ileum of unoperated control animals. Likewise, collagen synthesis was 52 ± 11 dpm CDP/μg DNA in intact colon in rats with an ileum anastomosis and 77 ± 8 dpm CDP/μg DNA in colon of unoperated controls.

Collagen synthesis was measured from 3 hours to 28 days after operation. In table 1 the average values for ileal anastomoses are given. Three hours after operation incorporation of label into CDP, expressed both on wet weight and DNA basis, was already significantly increased with respect to the unoperated controls. The relative collagen synthesis was significantly enhanced after 12 hours. From 3 hours to 4 days after operation both the absolute and the relative collagen synthesis rose steadily. Thereafter, synthetic activity started to fall: the average values in two 5 days-old anastomoses were 230 dpm CDP/mg wet weight, 132 dpm/μg DNA and 1.73 % relative collagen synthesis, respectively. After 4 weeks collagen synthesis in the anastomotic area had nearly returned to the pre-operative level, average values being not significantly different any more from those measured in the controls.

Table 1. Explants from anastomotic tissue were incubated for 3h with 4.5 μCi [^{3}H]proline. Results are expressed as dpm CDP and as percentage relative collagen synthesis. Data represent average values (±SD) from six animals. Differences between anastomoses and control intestine from unoperated rats was tested for significance using a one-sided Wilcoxon test: * $0.01 < p \le 0.05$; ** $p \le 0.01$

time after operation	CDP dpm/mg wet-weight	CDP dpm/μg DNA	% relative collagen synthesis
3h	177 ± 69**	57 ± 20*	0.36 ± 0.09
12h	262 ± 68**	71 ± 19**	0.43 ± 0.08*
1d	277 ± 87**	108 ± 52*	0.52 ± 0.10**
2d	310 ± 88**	101 ± 41*	0.61 ± 0.17**
3d	509 ± 82**	167 ± 64**	1.09 ± 0.21**
4d	687 ± 268**	300 ± 126**	2.42 ± 0.72**
7d	284 ± 104**	184 ± 120**	1.69 ± 0.67**
28d	109 ± 32	70 ± 32	0.54 ± 0.25
control	70 ± 18	41 ± 7	0.30 ± 0.04

The general pattern was the same in colon (table 2), although the increase in synthetic activity appeared delayed in comparison with ileum. The absolute collagen synthesis was significantly elevated after 12 hours, if expressed on a wet weight basis, and only after 2 days , if expressed on a DNA basis. Likewise, the relative collagen synthesis in the anastomotic area was significantly higher than in control intestine

from 24 hours after operation onwards. In colon, synthesis also seemed maximally stimulated after 4 days. The average values measured in two 5 days-old colonic anastomoses were 288 dpm CDP/mg wet weight, 151 dpm CDP/μg DNA and a relative collagen synthesis of 1.39 %, respectively. Values after 4 weeks were not significantly different from control values any more, except for the relative collagen synthesis which was still elevated.

Table 2. Explants from anastomotic tissue were incubated for 3h with 4.5 μCi [^{3}H]proline. Results are expressed as dpm CDP and as percentage relative collagen synthesis. Data represent average values ($\pm$SD) from six animals. Differences betwee anastomoses and control intestine from unoperated rats was tested for significance sing a one-sided Wilcoxon test: * 0.01< p ≤ 0.05 ; ** p ≤ 0.01.

time after operation	CDP dpm/mg wet-weight	CDP dpm/μg DNA	% relative collagen synthesis
3h	267 ± 77	73 ± 20	0.62 ± 0.14
12h	263 ± 35**	88 ± 16	0.64 ± 0.10
1d	370 ± 21**	123 ± 40	0.88 ± 0.14**
2d	399 ± 67**	126 ± 48*	0.93 ± 0.11**
3d	1002 ± 462**	253 ± 88**	2.65 ± 1.23**
4d	1086 ± 375**	373 ± 188**	2.87 ± 0.72**
7d	421 ± 148**	154 ± 55**	1.48 ± 0.51**
28d	192 ± 85	108 ± 36	1.01 ± 0.24**
control	188 ± 29	77 ± 8	0.52 ± 0.03

Next, we calculated at each time point the average ratio between the values measured in anastomoses and those observed in non-operated rats. Figure 1 shows that the increase in collagen synthesis, expressed on basis of either wet weight or DNA was always higher in ileum than in colon. For instance, maximal increase, with respect to normal value, was 9.8 ± 3.8 in ileum and 5.8 ± 2.2 in colon (if synthetic activities were expressed on a wet weight basis, figure 1A). In contrast, the stimulation of the relative collagen synthesis appeared comparable in both small and large bowel, certainly up to 2 days (figure 1C). Three days after operation, the average ratio in colon was even higher than in ileum, although at 4 and 7 days the effect was again the other way around.

In order to further substantiate the notion that anastomotic construction stimulates collagen synthesis more strongly in ileum than in colon, the relative increase in both segments was compared within each rat. To this purpose, the ratio between collagen synthesis in the ileal anastomosis and the average value found in the

non-operated controls was calculated. The same was done for the colonic anastomosis in the same animal. Subsequently, a final ratio was calculated between the ratio's found for ileal and colonic anastomoses. A ratio exceeding the value of 1 indicates that the collagen synthesis in the animal in question is enhanced more strongly in ileum than in colon. The data for all rats are represented in figure 2. Since the number of rats sacrificed at each time point is relatively small (6), the ileum-colon

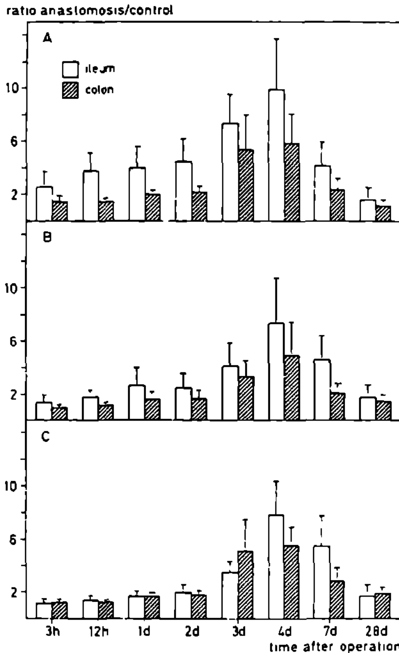

Figure 1. Relative increase of collagen synthesis in anastomotic explants from both ileum and colon. In each anastomosis collagen synthesis was measured as dpm CDP/mg wet weight (A), dpm CDP/μg DNA (B) or percentage relative collagen synthesis (C) and the ratio between this value and the average value in six unoperated controls was calculated. Values depicted represent the average ($\pm$SD) from six experimental animals.

difference is only significant (one-sided signed rank test) if a ratio above 1 is found in all 6 animals. For the synthetic activities, expressed on a wet weight basis (figure 2A) this was the case at 12 hours and 2 days after operation. Still, at the other time points a ratio above 1 was observed in 5 out of 6 animals. Altogether, the reverse - a ratio lower than 1, indicating a stronger stimulation in colonic anastomoses - was the case in only 6 out of 48 animals. This effect was even more pronounced if synthetic activity was expressed on the basis of DNA (figure 2B). Here, a significant effect was noted at 12 hours, 1 day, 4 days and 7 days after operation. A different picture emerged for the relative collagen synthesis (figure 2C): no consistent difference was noted between

ileum and colon. Stimulation of collagen synthesis in ileal anastomoses was consistently and significantly higher than in colonic anastomoses at 7 days after operation only.

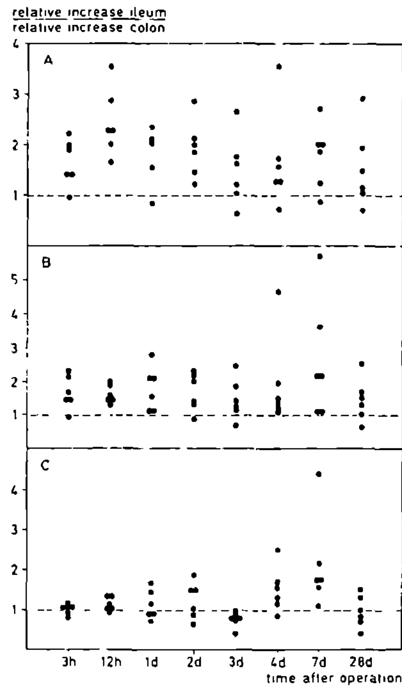

Figure 2. Ratio between ileal and colonic stimulation of anastomotic collagen synthesis. In each rat, the collagen synthesis in both ileal and colonic anastomotic explants was measured as dpm CDP/mg wet weight (A) , dpm CDP/μg DNA (B) and percentage relative collagen synthesis (C) and the ratio between these values and the average value in corresponding intestinal segments from six unoperated controls were calculated. The data depicted (each point representing one animal) were obtained by dividing the relative increase in the ileal anastomosis by the relative increase in the colonic anastomosis. The postoperative rise in collagen synthesis is significantly ($p \leq 0.05$) higher in ileal than in colonic anastomoses if all six values exceed 1 (one-sided signed rank test).

DISCUSSION

Collagen synthesis is an essential feature of anastomotic healing: the strength of the sutured intestinal wall has to be restored by newly-formed collagen fibrils. Inhibition of postoperative fibrillogenesis impairs the build up of anastomotic strength [11]. Disturbance of collagen synthesis in the anastomotic area will lead to loss of strength and probably to increased chances for anastomotic failure. The frequency rate of dehiscence of bowel anastomoses remains rather high [1]. Apparently, anastomoses are compromised regularly, even under optimal conditions, and a multitude of circumstances may aggravate the risk considerably [12], even to such extent that anastomotic construction has to be delayed. Since the underlying mechanisms are poorly understood, examination of the course and regulation of the primary molecular processes, such as the synthesis of collagen, is of major importance. Dehiscence of

bowel anastomoses appears mainly a problem in the large intestine [13]. Explanations for this difference between small and large bowel are speculative and it is unknown if molecular events which occur during healing differ between both bowel segments. If so, they might indicate how to proceed in order to improve colonic anastomotic repair. Indeed, our comparison of postoperative collagen synthesis in ileal and colonic anastomoses within the same animal shows that this process starts earlier and is more strongly stimulated in the small bowel.

We have employed explants of intestinal anastomoses and thus measured collagen synthesis in vitro. In an earlier communication we have shown that such a system is useful to asses changes which occur in vivo, such as induced by ageing [7]. The present results show that the considerable stimulation of anastomotic collagen synthesis, which had been shown to occur by means of in vivo measurements [5,6], is also found this way. The maximal stimulation observed, 9-fold in ileum and 6-fold in colon as compared to uninjured intestine, is similar in both types of experiments.

So far, no data had been published concerning anastomotic collagen synthesis during the first 2 days of the healing period. Clearly, synthesis is enhanced very quickly after wounding, certainly in ileum where incorporation of [^{3}H]proline into CDP has risen significantly already after 3 h. Since influx of fibroblasts, which are generally believed to be the source of collagen produced during wound healing, is only apparent 2 days after anastomotic construction [14] this means that resident cells respond to injury by increasing their capacity for collagen production. The fact that the relative collagen synthesis is also significantly enhanced in the early period indicates that this is not a general stimulation of protein synthesis but indeed a more specific response. It is impossible to decide from the present results if indeed fibroblasts are responsible for the collagen production. Recent experiments have shown that intestinal smooth muscle cells, isolated from human jejunum, are capable of synthesizing type I, III and V collagen [15,16]. The authors have suggested that these cells could play an important role in repair processes after damage and inflammation of the intestinal wall.

Next to the description of the early course of postoperative collagen synthesis in intestinal anastomoses, the comparison of this process in small and large bowel was the major goal of this study. So far, one report exists in the literature which attempts

such a comparison : in vivo collagen synthesis, expressed as specific activity (dpm [^{3}H]hydroxyproline /μmol hydroxyproline) and measured 4 days after operation only, was not significantly different in ileal and colonic anastomoses [17]. The same is probably true if we compare the average collagen synthesis, expressed as dpm/μg DNA, in ileal and colonic anastomoses at this time point (tables 1 and 2). However, such an approach does not take into consideration the fact [7] that basal synthesis in colon is significantly higher than in ileum. In order to allow comparison within each individual rat, we constructed both anastomoses in the same animal. Control experiments confirmed that anastomotic construction in the ileum does not affect collagen synthesis in unoperated colon and vice versa. If collagen synthesis is calculated as dpm CDP, expressed on the basis of wet weight or DNA, postoperative stimulation in ileum is higher than in colon in almost every animal examined (figure 2). Moreover, ileal synthesis is clearly stimulated at an earlier stage than synthesis in colon. For instance, incorporation of [^{3}H]proline into CDP and expressed per μg DNA, is significantly higher than in unoperated controls at 3 h after operation in ileum and at 48 h after operation in colon. Taken together, we believe that these results demonstrate that the ileum responds more quickly to wounding than the colon, at least as far as production of new collagen is concerned. This conclusion is in agreement with earlier studies [e.g. 4,8] which showed that the transient postoperative lowering of anastomotic hydroxyproline concentrations was less pronounced and more rapidly undone in the ileum.

The reasons why the small bowel reacts more rapidly in this respect than the large bowel remain as yet to be determined. The cells responsible for collagen synthesis, resident fibroblasts and/or smooth muscle cells [15,16], are stimulated by wounding, possibly by mediators released from platelets [18]. Somehow, colonic collagen-producing cells are less responsive to mediators released (e.g. platelet-derived growth factors) in the immediate post-operative period. Since the pre-operative level of collagen synthesis is higher in colon, it could be that the cells in question reside at a higher level of activity than those in ileum and therefore need a greater stimulus in order to increase their collagen production.

It is generally accepted that anastomotic collagen determines anastomotic strength. Next to synthesis, degradation of this matrix protein will affect wound

strength. Production of collagenase has been demonstrated immunohistochemically around colonic anastomoses [19] and we have found transiently increased extractable collagenolytic activity in the anastomotic area but no differences between ileal and colonic anastomoses [20]. While degradation of anastomotic collagen is probably limited under conditions, optimal for healing [21], it should be emphasized that this process could be enhanced by local or systemic factors and thus contribute to loss of strength.

In conclusion, we suggest that the differences in post-operative collagen synthesis, observed between ileal and colonic anastomoses constructed under normal conditions, may help to explain the differences observed between ileal and colonic anastomoses may help to explain the lower failure rate of anastomoses in the small intestine. Investigation of the regulation of collagen synthesis, either in tissue explants or on the cellular level, is needed in order to determine optimal conditions and allow improvement of this important aspect of repair, particularly in anastomoses which have to be constructed in high-risk conditions.

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CHAPTER 4

COLLAGEN SYNTHESIS THROUGHOUT THE UNINJURED AND ANASTOMOSED INTESTINAL WALL

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ABSTRACT:

Collagen synthesis has been measured throughout the intestinal tract of the rat, using an in vitro technique. In addition, the effect of anastomotic construction in either ileum or colon on collagen synthesis at specific distant locations has been investigated, both one and four days after operation.

Collagen synthesis is relatively uniform throughout the large bowel. However, in the small bowel, where synthesis is lower than in the large bowel, it is significantly higher in the most proximal and distal segments than in the intermediate tissue. Anastomotic construction in either part of the bowel strongly increases collagen synthesis at the immediate wound site. The synthetic response is not restricted to the anastomotic site but more generalized and, in the small bowel, to a certain extent specific for collagen. Anastomotic construction in the colon has only minor, though sometimes significant, effects on collagen synthesis in the ileum and vice versa.

SUMMARY:

Collagen synthesis is measured throughout the uninjured and anastomosed intestine of the rat. Significant differences are found between different sites. The increase after anastomotic construction is not restricted to the wound site but represents a more generalized response.

INTRODUCTION:

The strength of the bowel wall lies predominantly in the submucosa, which layer may be considered as the skeleton of the intestine providing it with structural integrity. The main structural component of this connective tissue layer is collagen: its main framework is composed of two arrays of collagen fibers running diagonally around the intestinal wall [1]. Thus, collagen is the single, most important molecule determining intestinal strength [2] and as such its metabolism is of particular interest. However, little is known about collagen synthesis throughout the uninjured intestine.

Resection and anastomosis of the intestine lead to profound changes in mechanical strength and in collagen levels in the wound area [3]. In this respect, differences exist between ileal and colonic anastomoses [4,5]. Postoperatively, anastomotic collagen synthesis is strongly enhanced, both in ileum and in colon [6,7].

Although Jönsson et al. [8] reported no differences in postoperative collagen synthesis between ileal and colonic anastomoses four days after operation, we have recently found significant differences during the first 48 hours [9]. Therefore, further characterization of the synthetic response in small and large intestine appears of interest. This is the more so since conflicting results have been published with regard to the postoperative collagen synthesis being a localized effect. While Stromberg and Klein found increased synthesis to be restricted to the immediate anastomotic area [10,11], Jönsson et al. [7] reported a far more general reaction.

We have been interested in comparing the healing response in ileum and colon because, clinically, anastomotic healing in the small bowel appears to be attended by far less complications. Comparison of the molecular events in experimental anastomoses may yield differences between both bowel segments and thus indicate ways to improve colonic healing. The present paper describes collagen synthesis - measured in vitro [12] - throughout the rat intestine, both before and after construction of an anastomosis in either ileum or colon.

MATERIALS AND METHODS

materials:

l-[2,3-^{3}H]Proline (300 Mci/mg) was purchased from Amersham International, England. Dulbecco's Modified Eagles Medium (DMEM) was obtained from Gibco, Breda, The Netherlands. Collagenase (type 7) and deoxyribonucleic acid (DNA, calf thymus) were obtained from Sigma, St-Louis, USA. The scintillation liquid used was picofluor-30 from Packard, Groningen, The Netherlands. All other reagents were of analytical grade (Merck, Darmstadt, Germany). Suture material used was ethilon 8x0 (Ethicon, Norderstedt, FRG).

animals:

Forty two male Wistar rats with a weight between 200 and 250 g were used. They were fed a standard diet (Hope Farms, Woerden, The Netherlands) and allowed water ad libitum. In three groups collagen synthesis was measured at 10 fixed sites along the intestinal tract: a control group of six unoperated animals, a group which received an ileal anastomosis (12 animals) and a group which received a colonic anastomosis (12 animals). Within the latter groups, six rats were sacrificed at either

one or four days after operation. In a fourth group the animals (12) each received both an ileal and colonic anastomoses and after either one or four days collagen synthesis was measured in the anastomotic area only.

<u>operative techniques:</u>

Surgery was performed under semi-sterile conditions using a Zeiss operating microscope. The rats were anaesthetized by an intraperitoneal injection with sodium pentobarbital. The abdomen was opened through a midline incision of approximately four cm. The ileum was transsected at 15 cm proximal to the ileal-caecal junction and an end-to-end anastomosis was constructed using eight single-layer inverting interrupted 8x0 Ethilon sutures. Subsequently (or alternatively), the descending colon was transsected three cm proximal to the peritoneal reflection and continuity was restored as described above. The abdomen was closed in two layers using silk for the fascia and staples for the skin.

<u>sampling of intestinal tissue:</u>

After sacrifiction by an overdose of sodium pentobarbital tissue samples were collected immediately for the measurement of collagen synthesis. The origin of the various samples is illustrated in figure 1. The entire intestine between stomach and peritoneal reflection was removed and the length of both small and large intestine was measured (large intestine: 10.5 ± 1.2 cm ; small intestine: 74.0 ± 5.0 cm; average value ± SD, n=30). The large intestine was divided into four equal parts which are referred to as A, B, C and D, respectively. From the small intestine five 3 cm-segments were resected: the first (E) was taken next to the caecum and the other four (F to I) each 15 cm in proximal direction. A final sample (K) was collected immediately distal to the stomach.

Anastomoses were constructed in either small intestine (ileum, segment F) or in large intestine (colon, segment B) or in both. In those animals where only one anastomosis was constructed the tissue containing the suture line (5 mm) was removed from the segment B or F before measuring collagen synthesis in that segment. Anastomotic collagen synthesis, i.e. in the 5 mm around the suture line, was measured separately.

<u>assay of collagen synthesis:</u>

Collagen synthesis was measured in tissue explants, according to a procedure

validated before for rat intestine [12] and intestinal anastomoses [9]. Tissue samples were cut into pieces of approximately 1-2 mm^2 and 50 mg wet weight for the uninjured tissue and 35-70 mg for the anastomotic tissue, was transferred to petri dishes (diameter 35 mm). The pieces were washed once with physiological salt solution and once with incubation medium (DMEM containing 50 μg/ml ascorbate and 250 μg/ml kanamycin). Subsequently, 1.5 ml incubation medium was added and the samples were incubated for 30 min at 37°C (95% air; 5% CO_2). The medium was then removed by suction and replaced with 1.5 ml incubation medium containing 4.5 μCi [2,3-^{3}H]proline. Incubation proceeded for 3 hr. All subsequent steps were carried out at 4°C. Both tissue and medium were transferred to a centrifugation tube and spun for 5 min at 2500 x g. The sediment was homogenized in 3.0 ml 50 mM Tris-HCl, pH 7.6, containing 25 mM ethylenediaminetetraacetic acid (EDTA), 10 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM proline. Trichloroacetic acid (TCA, final concentration 0.6 M) was added to the homogenate which was then centrifuged for 5 min at 2500 x g. The sediment was washed three times with 0.3 M TCA containing 1 mM proline.

The final sediment was dissolved in 0.75 ml 0.2 M NaOH and neutralized by the addition of 0.3 ml 1 M HEPES and 0.3 ml 0.15 M HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to determine proline incorporation into collagen 0.2 ml 20 mM Tris-HCl, pH 7.6, containing 50 mM $CaCl_2$ and 0.1 ml collagenase (chromatographically purified on a G200 gelfiltration column) were added to a 0.5 ml aliquot of the solubilized sample and the mixture was incubated for 5 h at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.6 M and 3 mM, respectively. After centrifugation (10 min; 14.500 x g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyzer. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase yielded the collagen specific incorporation, which will be referred to as collagenase digestible protein (CDP). Subtraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non collagenous protein (NCP). Incorporation into CDP and NCP is quantified on the basis of both mg wet weight used for

incubation and μg DNA present in the solubilized TCA sediment.

The relative collagen synthesis was calculated with the formula [13] that takes into account the enrichment of proline in collagen compared to other proteins :

$$\% \text{ relative collagen synthesis} = \frac{CDP}{(NCP \times 5.4) + CDP} \times 100\%$$

other procedures:

DNA was measured in the NaOH-solubilized TCA sediment, by the method of Burton [14] using calf thymus DNA as a standard. Statistical methods are mentioned with the results.

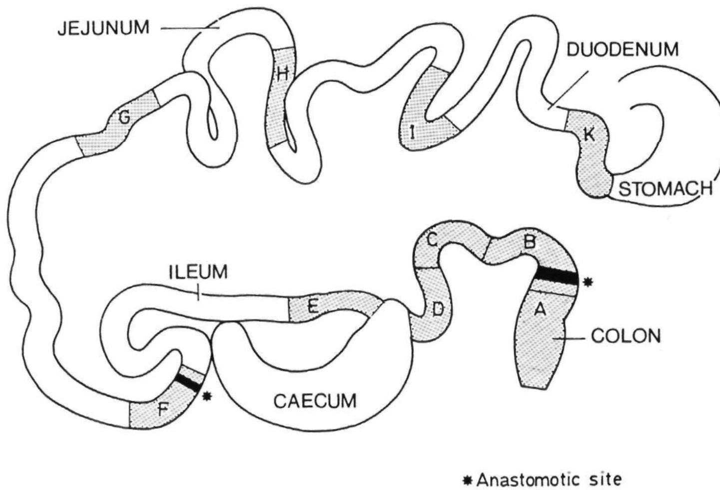

Figure 1. Schematic drawing of the intestinal tract of the rat, indicating sampling sites (A-K) and anastomotic locations.

RESULTS

Collagen synthesis was measured in biopsies collected throughout the intestine (cf. figure 1) from six uninjured animals. Both absolute, expressed on the basis of wet weight or DNA and relative collagen synthesis are given in table I. It is obvious that basal collagen synthesis is higher in colon than in ileum. Throughout the large intestine, variations are relatively small. Possibly, the synthetic activity in the most distal segment is somewhat higher than in the adjoining segment. More explicit differences are found throughout the small intestine. Clearly, collagen synthesis in the

Table I: Collagen synthesis in different segments from the large (A-D) and small bowel (E-K). Intestinal explants were incubated under standard conditions (50 mg, 3h) in the presence of 4.5 μCi [^{3}H]proline. Data represent the average values (±SD) from six separate experiments. Significant differences between the various segments are presented in the lower part of the table (one-sided Wilcoxon test: $0.02 \leq p \leq 0.05$).

segment:	dpm CDP/mg wet weight	dpm CDP/μg DNA	%RCS
A	213 ± 58	47 ± 10	0.85 ± 0.16
B	164 ± 70	41 ± 11	0.78 ± 0.13
C	176 ± 57	50 ± 11	0.78 ± 0.13
D	207 ± 49	51 ± 8	0.77 ± 0.15
E	97 ± 41	24 ± 8	0.44 ± 0.14
F	46 ± 22	16 ± 6	0.39 ± 0.09
G	30 ± 12	11 ± 4	0.36 ± 0.10
H	33 ± 16	12 ± 4	0.49 ± 0.17
I	55 ± 41	16 ± 7	0.49 ± 0.12
K	75 ± 33	22 ± 8	0.57 ± 0.10
ileum:	E>F; F>G; K>G E>G; K>H E>H E>I	E>G; F>G; K>G E>H; K>H E>I	E>F; G>H
colon:	A>B	none	A>D

biopsies obtained from the locations closest to the caecum (E) or stomach (K) is higher than in those collected in between. While this effect is observed for the absolute collagen synthesis, and then most explicitly if activity is expressed per mg wet weight, it is far less pronounced for the relative collagen synthesis (RCS) indicating that the non-collagenous protein synthesis also changes.

Collagen synthesis in intestinal anastomoses is considerably higher than in uninjured intestine. For animals with one anastomosis in either ileum or colon, table II shows a comparison of the collagen synthesis measured in the immediate (5 mm) anastomotic area with the average value for collagen synthesis in uninjured intestine collected at similar sites from six control rats. In the small intestine (ileum), both absolute and relative collagen synthesis are significantly enhanced already after one day. At this time, the increase is not yet significant in the large intestine (colon). Four days after operation, strong and specific stimulation of collagen synthesis is measured at both anastomotic sites. We also measured anastomotic collagen synthesis in rats which received an anastomosis in both ileum and colon. Table III shows that the stimulation, with respect to values in uninjured intestine, is similar to those observed in the animals with only one anastomosis.

Table II: Increased anastomotic collagen synthesis in animals with one anastomosis in both ileum and colon. Explants from anastomotic tissue were incubated for 3h with 4.5 μCi [^{3}H]proline. Results are expressed as the ratio (average ± SD, n=6) between values obtained from the anastomoses and the mean value found in similarly localized biopsies from 6 unoperated control animals.

	days after operation	ratio anastomosis/uninjured intestine CDP/mg wet weight	CDP/μg DNA	%RCS
Ileum	1	6.1 ± 3.1	2.6 ± 1.3	2.1 ± 1.5
	4	7.7 ± 4.4	5.8 ± 2.9	4.8 ± 2.2
Colon	1	1.8 ± 0.9	1.4 ± 0.5	1.2 ± 0.3
	4	6.1 ± 2.8	5.0 ± 1.7	4.0 ± 0.9

Table III: Increased anastomotic collagen synthesis in animals with an anastomosis in both ileum and colon. Explants from anastomotic tissue were incubated for 3h with 4.5 μCi [^{3}H]proline. Results are expressed as the ratio (average ± SD, n=6) between values obtained from the anastomoses and the mean value found in similarly localized biopsies from 6 unoperated control animals.

	days after operation	ratio anastomosis/uninjured intestine CDP/mg wet weight	CDP/μg DNA	%RCS
Ileum	1	4.0 ± 1.6	2.6 ± 1.3	1.7 ± 0.4
	4	9.8 ± 3.8	7.3 ± 3.3	8.1 ± 2.6
Colon	1	2.0 ± 0.3	1.6 ± 0.5	1.7 ± 0.3
	4	5.8 ± 2.2	4.8 ± 2.5	5.5 ± 1.4

The synthetic response after anastomotic construction is not restricted to the immediate wound area. Figure 2 depicts its effects on collagen synthesis calculated on a wet weight basis. One day after operation, an ileal anastomosis (upper panel) not only stimulates synthesis in the immediately adjacent tissue - segment F - but also in the more proximal small bowel segments G and H. No effects are noted in the large bowel. After 4 days, stimulation is highest in segment F but also significant in segments E, G and H. At this time, a small but significant increase is also observed in various parts of the large bowel. Construction of a colonic anastomosis (lower panel) leads, one day after operation, to enhanced synthesis only in the large bowel segment B. Surprisingly, the collagen synthesis has also risen in the ileal segments F and G. Four days after surgery, the stimulatory effect is more widespread through the colon, while stimulation in the ileum is limited to segment F.

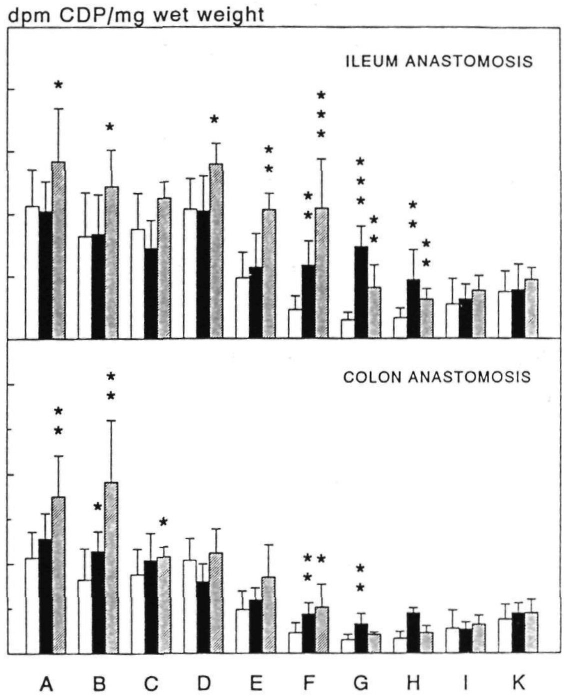

Figure 2. Effects of anastomotic construction on collagen synthesis, expressed per unit wet weight, throughout the intestine. Biopsy sites are depicted in figure 1. Open bars: values from non-operated animals; closed bars: values one day after anastomotic construction; striped bars: values four days after anastomotic construction. Anastomoses were constructed in either ileal segment F (upper panel) or colonic segment B (lower panel). Synthesis in segments F and B is measured after removal of the (5 mm) anastomotic segment. Results represent mean values ± SD from 6 animals. Differences between operated rats and uninjured controls are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

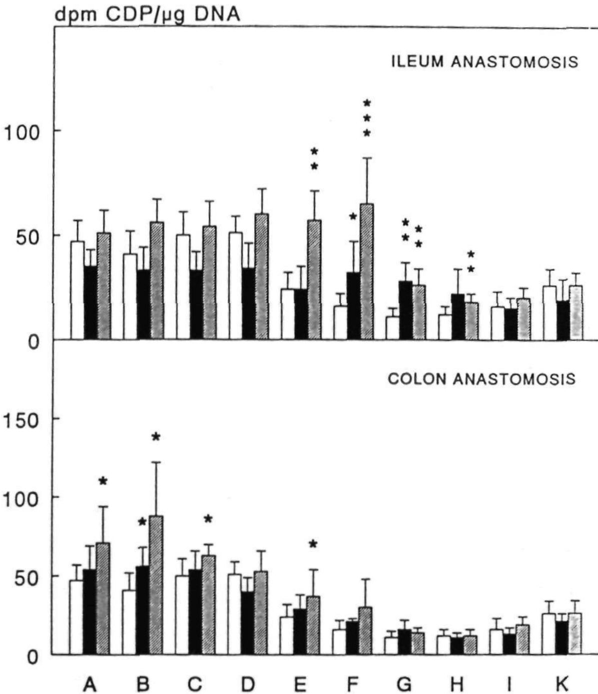

Figure 3. Effects of anastomotic construction on collagen synthesis, expressed per unit DNA, throughout the intestine. Biopsy sites are depicted in figure 1. Open bars: values from non-operated animals; closed bars: values one day after anastomotic construction; striped bars: values four days after anastomotic construction. Anastomoses were constructed in either ileal segment F (upper panel) or colonic segment B (lower panel). Synthesis in segments F and B is measured after removal of the (5 mm) anastomotic segment. Results represent mean values ± SD from 6 animals. Differences between operated rats and uninjured controls are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

Collagen synthesis has also been expressed on the basis of the amount of DNA, as a measure of the cellular content of the samples. Figure 3 shows that the generalized response becomes less pronounced if data are calculated this way. Construction of an ileal anastomosis does not significantly affect collagen synthesis in the colon at either time point but acts stimulatory through part of the small intestine, particularly if measured after 4 days. Likewise, 1 day after construction of an anastomosis in the colon synthesis is only enhanced in the area immediately next to the wound (segment B). After 4 days the effect, though minor, is also significant in the large bowel segments A and C and in the most distal segment (E) from the small bowel.

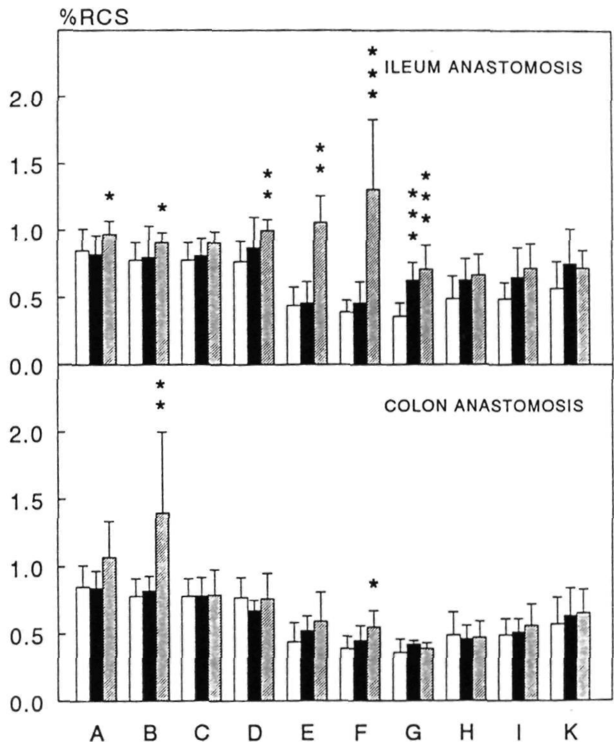

Figure 4. Effects of anastomotic construction on collagen synthesis, expressed as % relative collagen synthesis, throughout the intestine. Biopsy sites are depicted in figure 1. Open bars: values from non-operated animals; closed bars: values one day after anastomotic construction; striped bars: values four days after anastomotic construction. Anastomoses were constructed in either ileal segment F (upper panel) or colonic segment B (lower panel). Synthesis in segments F and B is measured after removal of the (5 mm) anastomotic segment. Results represent mean values ± SD from 6 animals. Differences between operated rats and uninjured controls are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

Finally, the effects on the relative collagen synthesis are depicted in figure 4. Anastomotic construction hardly affects the relative collagen synthesis as measured 1 day postoperatively, indicating that the effects described in figures 2 and 3 for this

time point probably represent a general increase of protein synthesis. However, 4 days after anastomotic construction in the ileum, the relative collagen synthesis is strongly enhanced in the small bowel segments E, F and G and, to a lesser extent, in the large bowel. At this time point, the response to a colonic anastomosis is limited to the surrounding area (B) and to ileal segment F.

DISCUSSION

Attempts to improve operative techniques and outcome of anastomotic wound healing must start with a better understanding of the molecular processes involved. One of the processes most crucial to the healing sequence is collagen synthesis [15], necessary to form new collagen fibrils which must restore strength to the intestinal wound.

So far there are no data available on collagen synthesis throughout the entire intestinal tract of the same species. Our results show that few differences exist in collagen synthesis throughout the large bowel. If any, synthesis in the most distal segment (A) would be highest. This is in agreement with results obtained by Törnquist et.al. [16], using an in vivo method to measure collagen synthesis. On the contrary, more explicit differences are found in the small bowel. Collagen synthesis is clearly elevated in the most proximal (K, duodenum) and most distal (E, terminal ileum) segments, if compared to sites in between. Altogether these data emphasize the necessity to standardize biopsy sites. For instance, if one aims to compare anastomotic collagen synthesis with synthesis in uninjured intestine, control values should be measured either in a segment resected peroperatively from the anastomotic site or in a similarly located segment from unoperated animals.

Anastomotic collagen synthesis is strongly enhanced as compared to synthesis in uninjured, nonoperated, rat intestine, already one day after operation. Using in vivo techniques, Zederfeldt's group has shown this to be true from two days after operation onwards [8]. Comparison of their results at four days after operation with the present data show that the degree of stimulation observed, is similar with both techniques. This result also supports the view that our in vitro system is appropiate to measure anastomotic collagen synthesis. Advantages of in vitro measurement are that far less labeled proline is required and that handling of radioactive animals can be

omitted.

Little is known about the effect of the construction of an anastomosis on the collagen synthesis in other parts of the gastro-intestinal tract. Stromberg and Klein have reported that there is no effect of anastomotic construction in either ileum and colon on the collagen synthesis in distant areas of the intestine [10,11]. However they measured collagen synthesis between seven and 21 days after operation, while anastomotic collagen synthesis is maximally enhanced during the first week [9,17]. In contrast to these results, which have been taken to indicate a very localized response to intestinal surgery, other studies [6,8,17] claim that the synthetic response is more general and also found at more distal sites along the intestine. Our results support this view.

One day after construction of a colonic anastomosis, collagen synthesis is only increased in the adjoining segment (B). After 4 days, enhanced synthesis is also seen more proximally (C, though not as far as D) and distally (A). These effects are only observed for the absolute collagen synthesis. The relative collagen synthesis remains unaffected at distant sites, indicating that the reaction represents a general effect on protein synthesis and is not specific for collagen. In the ileum, anastomotic construction leads to a stimulation of collagen synthesis both distal (E) and proximal (G,H) to the anastomotic site. Here, the relative collagen synthesis is also significantly enhanced in segments E and G. Thus, in the small bowel a specific increase in the synthesis of collagen is seen at locations at least 15 cm away from the injured area. In this respect, the intestine clearly differs from the skin where the effect of wounding appears to be very localized and restricted to the wound area [18,19].

Finally, we have observed that construction of an anastomosis in ileum has quantitatively small, but sometimes significant, effects on collagen synthesis in colon and vice versa. This phenomenon has also been reported by Jönsson et al., who described that construction of an ileal anastomosis stimulates collagen synthesis in the colon [7]. However, the effect is minor if compared to the direct local effect of anastomotic construction. For instance, construction of an ileal anastomosis increases absolute collagen synthesis, measured after four days and expressed on a wet weight basis, in colonic segment B by a factor 1.5 (figure 2). Construction of a colonic anastomosis only in segment B stimulates collagen synthesis in the anastomotic area

nearly 6-fold (table 2). If anastomoses are constructed in both ileum and colon, average stimulation in the colonic anastomosis is also 6-fold (table 3). Thus, if both an ileal and a colonic anastomosis are constructed in the same animal, an approach chosen by us to directly compare healing in small and large bowel [cf. references in 20], the effect of one anastomosis on the other would be negligible in this respect.

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CHAPTER 5

INTRAPERITONEAL CYTOSTATICS IMPAIR EARLY POST-OPERATIVE COLLAGEN SYNTHESIS IN EXPERIMENTAL INTESTINAL ANASTOMOSES

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SUMMARY:

Collagen synthesis in intestinal anastomoses has been measured in rats after in vivo administrated of cytostatics. The cytostatics were administered during five consecutive days either intravenously or intraperitoneally. On day three of the course the rats received both an ileal and a colonic anastomosis. The animals were sacrificed three and seven days after operation. The cytostatics regimen used was a combination of 5-fluorouracil, bleomycin and cisplatinum in a dose of 10, 2 and 0.35 mg/kg/day, respectively. In an additional group, a twice higher dose was given intraperitoneally.

Three days after operation a severe inhibition of the collagen synthesis was observed in all the cytostatics treated groups, both in ileum and in colon. The effects of intraperitoneal administration were much more pronounced than those observed after an equal dose given intravenously. Seven days after operation the collagen synthesis in the intravenously treated groups was restored to the level of the control group. However, in the intraperitoneal groups the collagen synthesis in ileal anastomoses was still inhibited.

Thus, cytostatics suppress collagen synthesis in intestinal anastomoses. The effect is more severe after intraperitoneal than after intravenous administration, confirming our earlier hypothesis that the former mode of administration comprises a higher risk for anastomotic integrity.

INTRODUCTION:

Surgery is the only effective treatment modality for malignant gastrointestinal tumours. However, local and/or regional recurrences following surgery remain a major problem in patients with colorectal carcinoma. The use of antineoplastic agents becomes increasingly important as a means to reduce recurrence rates. It could be argued that the most suitable time for adjuvant chemotherapy is during or immediately after tumor-reductive surgery (Martin, 1981, Mulder et al., 1983). Indications for administration of cytostatics in the peri-operative period stem from two facts: firstly, if the tumor cell population increases an even expanding number of drug-resistant phenotypes will develop which will become more difficult to eradicate (Goldie and Coldman, 1979, 1984) and secondly, surgery increases chemosensitivity (Gunduz et al., 1979). Thus there exists a biological rationale for the use of peri-

operative adjuvant chemotherapy.

If local and regional recurrences are accepted as important surgical treatment failures, the best adjuvant to prevent these recurrences would be early postoperative intraperitoneal chemotherapy (Cunliffe and Sugerbaker, 1989). An important mechanism which could account for the appearance of recurrences is that foci of neoplastic cells dislodged from the lateral margins of resection and lymphatic channels may be disseminated as micrometastases on the peritoneal surfaces and into the resection site. The tumour cells lost into the abdominal cavity might be trapped in fibrin and thus become protected from host defences and systemic chemotherapy. Therefore, both resection site and peritoneal surfaces should be fully exposed to intraperitoneal chemotherapy in the immediate postoperative period. This mode of administration would also allow higher local drug concentrations without exceeding the systemic toxicity level (Dedrick et al., 1978).

Chemotherapeutic agents do not selectively act on malignant cells but also have a negative influence on the healing of surgical wounds (Ferguson, 1982). We have shown very recently that this mode of treatment strongly impairs the development of strength in experimental intestinal anastomoses (de Roy van Zuidewijn et al., 1991). Thus, application of such therapy should proceed with caution since it will probably increase the chances for anastomotic failure. In order to find measures to prevent this harmful effect, its underlying mechanisms should be understood.

An important process in the wound healing sequence is collagen synthesis. The strength of both the intact and the anastomosed bowel wall is predominantly derived from collagen fibrils, located in the submucosa (Thomson et al., 1987, Graham et al., 1988). We have established a technique to measure collagen synthesis in intestinal explants (Martens and Hendriks, 1989). Using this technique, it can be demonstrated that anastomotic collagen synthesis increases shortly after operation and remains strongly elevated during the first postoperative week (Martens and Hendriks, 1991). The current experiment was performed to investigate if a 5-day course of bleomycin, 5-fluorouracil and cisplatin, administered either intravenously or intraperitoneally, would effect postoperative collagen synthesis in intestinal anastomoses constructed on the third day of cytostatics administration.

MATERIALS AND METHODS

Materials:

l-[2,3-^{3}H]Proline (300 mCi/mg) was purchased from Amersham International, England. Dulbecco's Modified Eagles Medium (DMEM) was obtained from Gibco, Breda, The Netherlands. Collagenase (type 7) and deoxyribonucleic acid (DNA, calf thymus) were obtained from Sigma, St-Louis, USA. The scintillation liquid used was picofluor-30 from Packard, Groningen, The Netherlands. Kanamycin was obtained from Gist-Brocades,Delft, The Netherlands. The cytostatics used were 5-fluorouracil (Roche Laboratories), bleomycin (Lundeck) and cisplatinum (Bristol-Meyers). All other reagents were of analytical grade (Merck, Darmstad, Germany). Suture material used was ethilon 8x0 (Ethicon, Norderstedt, Germany).

animals:

Fifty-four male wistar rats with a weight of 200 - 250 g were used. They were fed a standard diet (Hope Farms, Woerden, The Netherlands) and allowed water ad libitum. Four groups were formed each consisting of 12 animals: a control group, a group which received the cytostatics intravenously (IV), and two groups which received intraperitoneal cytostatics at two different dosages (IP1 and IP2, respectively). Within each group, six rats were sacrificed at both three and seven days after operation. The remaining six animals served as non-operated controls.

cytostatics treatment:

The cytostatics regimen consisted of a combination of 5-fluorouracil, bleomycin and cisplatinum in concentrations of 10, 2 and 0.35 mg/kg/day, respectively (IV and IP1 groups; de Roy van Zuidewijn et al., 1986). The dosage in the IP2 group was twice as high. The compounds were dissolved in saline and administered once daily for five consecutive days, either via the penis vein (2 ml: IV group) or directly into the peritoneal cavity (10 ml: IP groups). Since administration of saline alone does not affect healing, animals in the control group received no saline either way. On day three of the cytostatics course the rats were operated and each received both an ileal and colonic anastomosis.

operative techniques:

Surgery was performed under semi-sterile conditions using a Zeiss operating microscope. The rats were anaesthetized by an intraperitoneal injection of sodium

pentobarbital. The abdomen was opened through a midline incision of approximately four cm. The ileum was transsected at 15 cm proximal to the ileal-caecal junction and an end-to-end anastomosis was constructed using eight single-layer inverting interrupted 8x0 Ethilon sutures. Subsequently, the descending colon was transsected three cm proximal to the peritoneal reflection and continuity was restored as described above. The abdomen was closed in two layers using silk for the fascia and staples for the skin.

After three or seven days the animals in each group were sacrificed by an intraperitoneal overdose of sodium pentobarbital and the anastomotic segments were resected, opened longitudinally and washed twice with physiological salt solution.

assay of collagen synthesis:

Collagen synthesis was measured in tissue explants, according to a procedure validated before for rat intestinal anastomoses (Martens and Hendriks 1989, 1991). The anastomosis proper (± 2 mm left and right of the transection line) was isolated and cut into pieces of approximately 1-2 mm^2. Two equal samples, 35-70 mg wet weight, were transferred to petri dishes (diameter 35 mm). The pieces were washed once with physiological salt solution and once with incubation medium (DMEM containing 50 μg/ml ascorbate and 250 μg/ml kanamycin). Subsequently, 1.5 ml incubation medium was added and the samples were incubated for 30 min at 37°C (95% air; 5% CO_2). The medium was then removed by suction and replaced with 1.5 ml incubation medium containing 4.5 μCi [2,3-^{3}H]proline. Incubation proceeded for 3 hr. All subsequent steps were carried out at 4°C. Both tissue and medium were transferred to a centrifugation tube and spun for 5 min at 2500 x g. The sediment was homogenized in 3.0 ml 50 mM Tris-HCl, pH 7.6, containing 25 mM ethylene-diamine-tetra-aceticacid (EDTA), 10 mM N-ethylmaleimide (NEM), 1 mM phenyl-methyl-sulfonylfluoride (PMSF) and 1 mM proline. Trichloroacetic acid (TCA) was added (final concentration 0.6M) to the homogenate which was then centrifuged for 5 min at 2500 x g. The sediment was washed three times with 0.3M TCA containing 1 mM proline.

The final sediment was dissolved in 0.75 ml 0.2 M NaOH and neutralized by the addition of 0.3 ml 1 M HEPES and 0.3 ml 0.15 M HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In

order to determine proline incorporation into collagen 0.2 ml 20 mM Tris-HCl, pH 7.6, containing 50 mM $CaCl_2$ and 0.1 ml collagenase (chromatographically purified on a G200 gelfiltration column) were added to a 0.5 ml aliquot of the solubilized sample and the mixture was incubated for 5 hr at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.6M and 3 mM, respectively. After centrifugation (10 min; 14.500 x g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyzer. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase yielded the collagen specific incorporation, which will be referred to as collagenase-digestible protein (CDP). Subtraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non-collagenous protein (NCP). Incorporation into CDP and NCP is quantified on the basis of both mg wet weight used for incubation and µg DNA present in the solubilized TCA sediment.

The relative collagen synthesis was calculated with the formula (Peterkofsky et al., 1981) that takes into account the enrichment of proline in collagen compared to other proteins :

$$\text{\% relative collagen synthesis} = \frac{CDP}{(NCP \times 5.4) + CDP} \times 100\%$$

<u>other procedures:</u>

DNA was measured in the NaOH-solubilized TCA sediment, by the method of Burton (Burton, 1956) using calf thymus DNA as a standard. Statistical methods are mentioned with the results.

RESULTS:

The collagen synthesis in intestinal anastomoses is strongly enhanced during the first week after operation. Table I shows both collagen and non-collagen synthesis in ileal anastomoses, as compared to uninjured intestine. The synthetic rate, measured as radioactivity incorporated into the CDP fraction, is significantly increased both three and seven days after operation. If synthesis is expressed on the basis of wet weight, the increase is eight-fold after three days and four-fold after one week. Synthesis of non-collagenous proteins is hardly affected by operation. The only significant

Table I: Collagen and non-collagen protein synthesis in uninjured and anastomotic ileal tissue. Explants from normal and anastomotic ileal tissue were incubated for 3h with 4.5 μCi [^{3}H]proline. Collagen synthesis is expressed as radioactivity in collagenase digestible protein (CDP) and as percentage relative collagen synthesis (RCS). Non-collagen synthesis is expressed as radioactivity into non-collagenous protein (NCP). Data represent average values (±SD) from six animals. Differences between anastomoses and control intestine from unoperated rats are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$

	uninjured intestine	3 days after operation	7 days after operation
CDP:			
- dpm/mg wet weight	70 ± 18	509 ± 82**	284 ± 104**
- dpm/µg DNA	41 ± 7	167 ± 64**	184 ± 120**
NCP:			
- dpm/mg wet weight	4420 ± 820	8591 ± 1471**	2841 ± 449
- dpm/µg DNA	2563 ± 343	2948 ± 1544	1794 ± 778
%RCS	0.30 ± 0.04	1.09 ± 0.21**	1.68 ± 0.67

Table II: Collagen and non-collagen protein synthesis in uninjured and anastomotic colonic tissue. Explants from normal and anastomotic colonic tissue were incubated for 3h with 4.5 μCi [^{3}H]proline. Collagen synthesis is expressed as radioactivity in collagenase digestible protein (CDP) and as percentage relative collagen synthesis (RCS). Non-collagen synthesis is expressed as radioactivity into non-collagenous protein (NCP). Data represent average values (±SD) from six animals. Differences between anastomoses and control intestine from unoperated rats are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$.

	uninjured intestine	3 days after operation	7 days after operation
CDP:			
- dpm/mg wet weight	188 ± 29	1002 ± 462**	421 ± 148**
- dpm/µg DNA	77 ± 8	167 ± 88**	154 ± 55**
NCP:			
- dpm/mg wet weight	6323 ± 1352	6637 ± 1371	4798 ± 934
- dpm/µg DNA	2622 ± 385	1851 ± 943	1778 ± 376
%RCS	0.52 ± 0.03	2.65 ± 1.23**	1.48 ± 0.51**

difference between anastomoses and uninjured intestine is found after three days and when NCP synthesis is calculated on the basis of wet weight. As a consequence, the percentage relative collagen synthesis is also significantly higher in anastomoses than in normal intestine. A similar picture emerges for colonic explants (table II), although the stimulation of the absolute collagen synthesis appears slightly less pronounced. The increase in CDP, expressed on the basis of tissue weight, is five and two fold after three and seven days, respectively. Here, no significant changes are found in the synthetic rate of non-collagenous proteins.

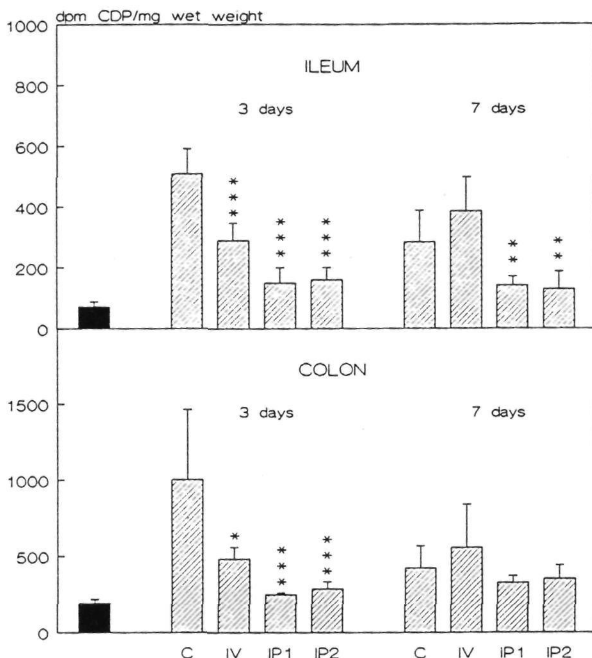
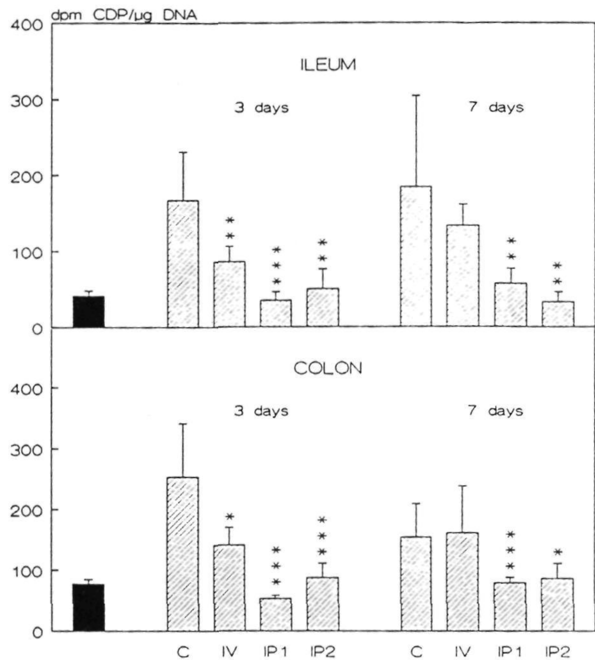


Figure 1. Effects of cytostatics on collagen synthesis, expressed per unit wet weight, in intestinal anastomosis. Rats were untreated (control group: C) or treated with cytostatics, either intravenously (IV) or intraperitoneally (IP; dose in the IP2 group twice higher than in IV and IP1 groups). Black bars represent values in uninjured intestine from non-operated animals. Average values (+SD) from 6 animals are given. Differences between control and each cytostatics group are tested for significance using a one-sided Wilcoxon test: * $0.01< p \leq 0.05$; ** $0.001< p \leq 0.01$; *** $p \leq 0.001$.

Administration of cytostatics severely affects postoperative collagen synthesis. Figure 1 depicts the changes in CDP synthesis, expressed on the basis of wet weight. Three days after operation synthesis is significantly lower in all cytostatics groups than in the control group. In ileum, values are suppressed by 43% in the IV group and by 71 and 68% in the IP1 and IP2 group, respectively. Synthetic rates in colonic anastomoses are affected similarly. Seven days after operation, the effect of peri-operative cytostatics persists only in the ileal anastomoses from the animals which had received the drugs intraperitoneally.

The absolute collagen synthesis in the various groups, expressed on the basis of the amount of DNA present, is depicted in figure 2. Again, incorporation of radioactivity into CDP is strongly inhibited three days postoperatively if cytostatics are administered, either intravenously or intraperitoneally, in the peri-operative period. In the IV group the inhibition is no longer apparent after seven days, while at this time point values in the IP groups remain significantly below those in the control group, both in ileum and in colon.

Figure 2. Effects of cytostatics on collagen synthesis, expressed on a DNA basis, in intestinal anastomosis. Rats were untreated (control group: C) or treated with cytostatics, either intravenously (IV) or intraperitoneally (IP; dose in the IP2 group twice higher than in IV and IP1 groups). Black bars represent values in uninjured intestine from non-operated animals. Average values (+SD) from 6 animals are given. Differences between control and each cytostatics group are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.



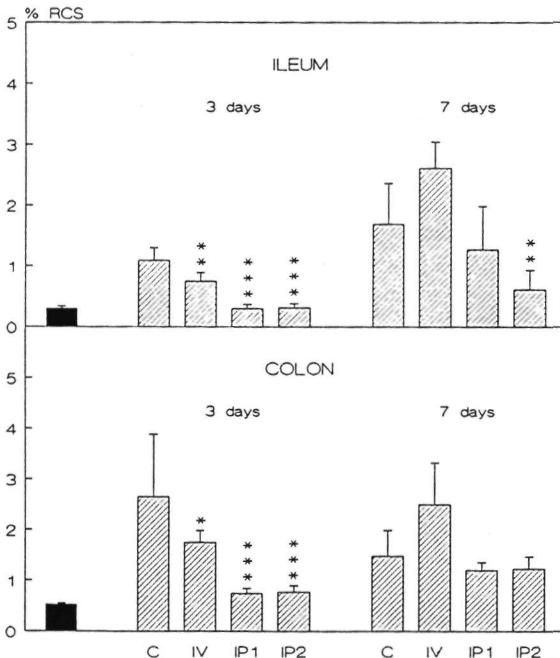


Figure 3. Effects of cytostatics on relative collagen synthesis in intestinal anastomosis. Rats were untreated (control group: C) or treated with cytostatics, either intravenously (IV) or intraperitoneally (IP; dose in the IP2 group twice higher than in IV and IP1 groups). Black bars represent values in uninjured intestine from non-operated animals. Average values (+SD) from 6 animals are given. Differences between control and each cytostatics group are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

Figure 3 shows the data for the percentage relative collagen synthesis. Collagen synthesis, as percentage of the total protein synthesis, after three days is significantlylower in all cytostatics groups than in the control group. In ileum, the relative collagen synthesis decreases by 32% in the IV group and by 74% in both IP groups. The suppression does not persist at seven days, with the exception of the ileal anastomoses from the IP2 group. Thus, both absolute and relative anastomotic collagensynthesis are inhibited to approximately the same extent. This can only be the case if the synthesis of non-collagenous proteins remains unaffected by the administration of cytostatics, which fact is illustrated in figure 4. Indeed, no differences between the control group and the cytostatics groups are observed, with respect to the incorporation of radioactivity into NCP, at three days after operation.

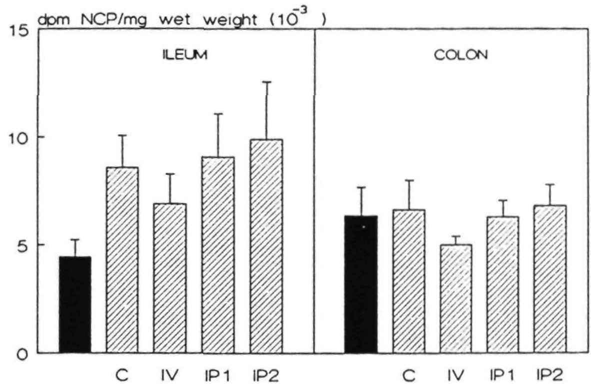


Figure 4. Effects of cytostatics on non-collagen protein synthesis, in 3 days-old intestinal anastomoses. Rats were untreated (control group: C) or treated with cytostatics, either intravenously (IV) or intraperitoneally (IP; dose in the IP2 group twice higher than in IV and IP1 groups). Black bars represent values in uninjured intestine from non-operated animals. Average values (+SD) from 6 animals are given.

It is clear from the preceding data that there exist profound differences between control and cytostatics groups. However, there also exist significant differences between groups depending on the mode of administration of the drugs. Statistical comparison of anastomotic collagen synthesis in the various cytostatics groups shows that the suppressive effects are, almost without exception, greater after intraperitoneal administration than after intravenous administration. This holds for both ileal (table III) and colonic (table IV) anastomoses. Although in general both IP groups react similarly, in a few cases differences are also found between groups after low and high doses of intraperitoneal cytostatics. At seven days after operation, in ileum, suppression of both absolute collagen synthesis, calculated on the basis of

DNA, and relative collagen synthesis is significantly more explicit in the IP2 than in the IP1 group. In colon, the only significant difference between both IP groups is for the absolute collagen synthesis, expressed as CDP/μg DNA, after three days.

Table III: Significant differences with respect to ileal anastomotic collagen synthesis between the three groups treated with cytostatics. Collagen synthesis in ileal anastomotic tissue was measured after incorporation of [^{3}H]-proline. Rats were treated in vivo with cytostatics, either intravenously (IV) or intraperitoneally (IP). The concentration of cytostatics in the IV and IP1 group is equal, whereas the concentration in the IP2 group is twice as high. Differences between the groups are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

Collagen synthesis expressed as:	3 days after operation	7 days after operation
CDP/mg wet weight	IP1 < IV**	IP1 < IV***
	IP2 < IV**	IP2 < IV**
CDP/μg DNA	IP1 < IV***	IP1 < IV**
	IP2 < IV**	IP2 < IV**
		IP2 < IP1*
%RCS	IP1 < IV***	IP1 < IV*
	IP2 < IV***	IP2 < IV**
		IP2 < IP1*

Table IV: Significant differences in colonic anastomotic collagen synthesis between the three groups treated with cytostatics. Collagen synthesis in colonic anastomotic tissue was measured after incorporation of [^{3}H]-proline. Rats were treated in vivo with cytostatics,either intravenously (IV) or intraperitoneally (IP). The concentration of cytostatics in the IV and IP1 group is equal, whereas the concentration in the IP2 group is twice as high. Differences between the groups are tested for significance using a one-sided Wilcoxon test: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

Collagen synthesis expressed as:	3 days after operation	7 days after operation
CDP/mg wet weight	IP1 < IV***	IP1 < IV**
	IP2 < IV***	
CDP/μg DNA	IP1 < IV***	IP1 < IV**
	IP2 < IV**	IP2 < IV**
	IP1 < IP2***	
%RCS	IP1 < IV***	IP1 < IV***
	IP2 < IV***	IP2 < IV**

DISCUSSION:

Anastomotic dehiscence is a most serious complication after surgery of the gastro-intestinal tract. This phenomenon is still seen rather frequently, even in cases of elective surgery under optimal conditions (Fielding et al., 1980). Since antineoplastic

agents interfere with wound healing (Ferguson, 1982), the use of these drugs in the peri-operative period is thought to be a danger to anastomotic integrity. Indeed, our previous experiments confirmed that a five day course of 5-fluorouracil, bleomycin and cisplatinum, given intravenously, lowers the mechanical strength of ileal anastomoses constructed on the third day (de Roy van Zuidewijn et al., 1986, 1988). In addition, intraperitoneal administration of the same regimen resulted in impaired strength of both ileal and colonic anastomoses (de Roy van Zuidewijn et al., 1991). The same was found to be true for jejunal anastomoses in rats treated with mitomycin-C (Fumagalli et al., 1991).

The bowel wall derives its strength mainly from collagen fibrils located in the submucosa (Thomson et al., 1987, Gabella, 1987). During the first week after surgery anastomotic collagen levels change, supposedly as the result of collagen degradation and synthesis (Hunt et al., 1980). While early anastomotic strength probably depends on the suture holding capacity of the existing collagen fibrils (Högström et al., 1985), newly-formed fibrils should restore pre-operative strength to the sutured bowel. In vivo measurements have established the occurrence of increased synthetic activity around experimental anastomoses in ileum (Jönsson et al., 1987) and colon (Jiborn et al., 1980). We have recently confirmed and extended these observations (Martens and Hendriks, 1991), using an in vitro technique for measuring collagen synthesis (Martens and Hendriks, 1989) also employed in the present study.

Anastomotic collagen synthesis is strongly enhanced at the time points chosen for this study (tables I and II). This effect is probably rather specific for collagen since the production of non-collagenous protein hardly changes and, as a result, the percentage relative collagen synthesis increases. We have expressed the results both on the basis of wet weight and on the basis of the amount of DNA present. The incorporation of [^{3}H]proline into CDP, expressed per unit DNA, also increases, but to a lesser extent than incorporation expressed per unit weight, in particular three days after operation. This indicates that the rise in production of collagen in the wound area is caused not only by an increased number of inflammatory cells, in particular fibroblasts (Hesp et al., 1985), but also by a stimulation of the synthetic rate per cell.

Early anastomotic collagen synthesis is strongly reduced if surgery takes place

in the period that the animals receive cytostatics. This phenomenon is observed in both ileum and colon and appears to be specific since the production of non-collagenous protein remains unaffected. Intravenous cytostatics inhibit collagen synthesis to a lesser degree than equal doses given intraperitoneally. The effects are also more transient since seven days after operation no differences are noted anymore between the control and IV groups, while significant inhibition is still apparent in the IP groups, particularly in ileum. Apparently, intravenous administration results in systemic dilution and thus intraperitoneal administration yields a higher concentration at the anastomotic site. Thus, while intraperitoneal cytostatics may be less harmful in terms of systemic toxicity, this route of administration is more detrimental with respect to collagen synthesis which process is crucial for anastomotic repair. No clinical data are available to support our experimental results. While intravenous administration of 5-fluorouracil alone in the peri-operative period does not appear to increase the incidence of anastomotic leakage (Taylor et al., 1985; Klausner et al., 1986), no results are known as yet for intraperitoneal chemotherapy. Our present data, together with those published before (de Roy van Zuidewijn et al., 1991) indicate that the latter mode of administration would carry greater risks for anastomotic integrity.

On the whole, we found few differences between the IP1 and IP2 groups. Doubling the dose does not result in further inhibition of either absolute or relative collagen synthesis as measured three days after operation. Presumably, there exists a basal level of synthesis, almost equal to that observed in the uninjured intestine, which remains unaffected by chemotherapy. One effect of the higher dose is that the inhibitory effect is prolonged: a significantly stronger inhibition is noted in seven days old ileal anastomoses from the IP2 group. This could be explained by more sustained inhibitory concentrations, although it remains puzzling why this does not affect the colonic anastomoses.

In earlier studies we found that peri-operative administration of cytostatics induced a decreased anastomotic collagen content (de Roy van Zuidewijn et al., 1986, 1988, 1991). Although we have not measured collagen degradation under these conditions, the present results strongly suggest that they are caused by a severe inhibition of the synthesis. In one of these studies (de Roy van Zuidewijn et al., 1991) we reported that different doses of intraperitoneal cytostatics, equal to those used in

the present study, had divergent effects on the mechanical strength of intestinal anastomoses: the higher dose induced significantly more loss of strength. No such clear differences are found between the IP1 and IP2 groups with respect to collagen synthesis. This fact supports the hypothesis (Hendriks and Mastboom, 1990) that the amount of collagen present is not the only, and perhaps not the most decisive, factor deciding anastomotic strength. Certainly the quality of the collagen, in particular its intra- and intermolecular crosslinks, are important. It could very well be that increasing doses of cytostatics interfere with crosslinking without affecting the rate of synthesis of collagen monomers.

Although it has been shown that endothelial cells from the rat small intestine (Quaroni and Trelstad, 1980) and smooth muscle cells from the human jejunum are capable of synthesizing collagen (Graham et al., 1987), it seems likely that the fibroblasts are primarily responsible for the increased collagen production in the wound area. The number of fibroblasts is strongly increased from two days after operation onwards (Hesp et al., 1985). The mechanisms which are responsible for the suppression of collagen synthesis are as yet unknown. Fibroblast chemotaxis could be affected, either directly or through inhibition of macrophage function. We have found histological evidence that the number of fibroblasts in ileal anastomoses is decreased by cytostatics [de Roy van Zuidewijn et al, submitted]. However, cytostatics could also interfere directly with fibroblast protein synthesis. It has been shown that doxorubicin inhibits collagen synthesis in fibroblast cultures (Sasaki et al., 1987).

Thus, this study indicates that the cytostatics regimen used severely inhibits anastomotic collagen synthesis, which effect probably causes the delay in the development of mechanical strength, reported before. The effects of intravenous administration are less severe and less persistent than those of equivalent doses given intraperitoneally. We believe that the present data support the notion that peri-operative intraperitoneal cytostatics are harmful to anastomotic repair in the intestine.

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CHAPTER 6

INHIBITION OF BASAL AND TGFß-INDUCED FIBROBLAST COLLAGEN SYNTHESIS BY ANTINEOPLASTIC AGENTS

Implications for wound healing

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Br. J. Cancer; in press

SUMMARY

Antineoplastic drugs, given in the perioperative period, are thought to be a hazard to wound repair. Since fibroblast collagen synthesis is crucial to healing, we examined the effects of bleomycin, cisplatin and 5-fluorouracil on collagen synthesis in confluent cultures of human fibroblasts from human colon and skin. The drugs were added in final concentrations between 0.1 and 50 μM.

Bleomycin did not affect collagen synthesis in colon fibroblasts but inhibited synthesis in skin fibroblasts. Collagen synthesis in colon fibroblasts was strongly, and specifically, inhibited by cisplatin while synthesis in skin fibroblasts was affected only slightly. 5-Fluorouracil had no effect whatsoever on the collagen synthetic capacity in either colon or skin fibroblasts. If skin fibroblasts were cultured in the presence of transforming growth factor ß (TGFß), the antineoplastic agents inhibited the TGFß-stimulated collagen synthesis at far lower concentrations than those needed to suppress non-stimulated synthesis. This effect was not observed in fibroblasts from colon.

The possible implications of these observations, as pertain to the use of perioperative chemotherapy, are discussed. It is suggested that the data support the contention that 5-fluorouracil is relatively harmless for intestinal healing.

INTRODUCTION

At present, surgery remains the only effective treatment modality for patients with malignant gastro-intestinal tumors. However, the occurrence of local and/or regional recurrences constitutes a major problem in the management of such patients. Recurrence rates may be reduced by antineoplastic therapy. Adjuvant treatment for colorectal cancer continues to center on the use of 5-fluorouracil and regimens that include 5-fluorouracil offer the greatest hope for patients with this malignancy [1]. It can be argued that the most suitable time for such therapy would be during or immediately after tumor-reducing surgery [2]. Since it is generally accepted that most antineoplastic agents used in the perioperative period will impede tissue repair [3], the healing of intestinal anastomoses appears to be at risk after administration of this class of compounds [4]. Indeed, studies from our laboratory have shown that a combination of bleomycin, cisplatin and 5-fluorouracil, given once a day over five

consecutive days, severely impairs the development of strength in experimental intestinal anastomoses constructed on the third day of the cytostatic regimen [5,6]. In contrast, administration of 5-fluorouracil alone appears to be less detrimental [7, de Waard et al., manuscript in preparation].

The strength of the intact and the anastomosed bowel wall is derived from collagen fibrils. Normal anastomotic healing is characterized by a strongly enhanced collagen synthetic activity [8,9]. The loss of strength encountered after administration of the cytostatics mixture mentioned above is attended by diminished deposition of collagen in the wound area as a result of a massive inhibition of the collagen synthetic capacity [10]. Fibroblasts are the major source of newly-formed collagen in a healing wound. Thus, inhibition of wound collagen synthesis by antineoplastic agents may be the result of their effects on fibroblast chemotaxis and proliferation, either direct or indirect through effects on macrophages, which cells play a pivotal regulatory role in the healing sequence [11]. This way, the diminished protein synthesis would simply be the result of a reduced presence of fibroblasts in the wound area. In addition, these drugs may directly affect fibroblast protein synthesis. Very few studies are known which report on the specific effects of antineoplastic agents on fibroblast collagen synthesis. We have examined if, and to what extent, bleomycin, cisplatin and 5-fluorouracil inhibit fibroblast collagen synthesis and if these effects are specific for collagen. For this purpose, we have used fibroblasts from both human colon and skin since we found recently that collagen production in these cells may react differently to various stimuli [12].

We have also investigated the effects of the three antineoplastic agents on fibroblast collagen synthesis measured after addition of transforming growth factor ß (TGFß). TGFß enhances fibroblast collagen production [13] and, if applied topically, promotes wound healing [14]. Its intrinsic role in the repair process is indicated by its transient and localized expression in healing wounds [15,16]. Therefore, interference with TGFß-induced fibroblast collagen synthesis may seriously affect wound repair.

MATERIALS AND METHODS

Materials:

All supplies for cell culture were purchased from Life Technologies (Breda, The Netherlands). The cytostatics used were bleomycin (Lundbeck, Amsterdam, The Netherlands), cisplatin (Lederle, Etten-Leur, The Netherlands) and 5-fluorouracil (Roche, Mijdrecht, The Netherlands). TGFß1 from bovine bone was a gift from dr. G. Ksander (Celtrix Labs, Palo Alto, USA). L-[2,3-^{3}H]Proline (1,63 TBq/mmol) and [6-^{3}H]thymidine (963 GBq/mmol) were purchased from Amersham International, England. Collagenase (type VII) was obtained from Sigma (St. Louis, USA). All other reagents were of analytical grade (Merck, Darmstadt, Germany).

Cell culture:

Normal human colon fibroblasts (HCF) were obtained from the American Type Culture Collection (CRL-1459). Human skin fibroblasts (HSF) were obtained from explants of skin biopsies of a healthy adult. Both the HSF and HCF were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 10% fetal calf serum (FCS) at 37°C in a 5% CO_2, 95% air humidified atmosphere. Cells were used between the third and tenth passage.

Assay of fibroblast proliferation:

Freshly trypsinized fibroblasts were plated in 96-well microtiter plates at a density of approximately $5x10^4$ cells/well in 0.1 ml DMEM plus 10% FCS. After a 4 h incubation the medium was replaced by 0.1 ml DMEM and the cells were incubated for a further 18 h. Subsequently, the medium was replaced again by 0.1 ml DMEM plus 10% FCS and incubation continued for another 48 h. At the end of this period antineoplastic agents (10 μl, dissolved in DMEM) were added, after 6 h followed by 0.5 μCi ^{3}H-thymidine. After a final incubation period of 18 h, the medium was removed and the cells were trypsinized, harvested on a filter using a cell harvester (LKB Wallag) and the incorporation of thymidine was counted.

Assay of fibroblasts collagen production:

Collagen production by steady state, visually confluent fibroblasts was assessed over a 24-h period by [^{3}H]proline incorporation into collagenous protein.

Freshly trypsinized fibroblasts were plated in 6-well 9.6 cm^2 tissue culture plates at a

density of approximately $1.5x10^5$ cells/well in 2 ml DMEM plus 10% FCS. Three days after plating the medium was removed and replaced by the same medium or with DMEM without serum. In the latter case the wells were first washed twice with phosphate buffered saline (PBS). Twenty four hours later the medium was replaced by the same medium plus ascorbic acid (50 μg/ml), ß-aminopropionitrile (50 μg/ml) and 2 μCi/ml [2,3-^{3}H]proline for the final 24 h of culture. The antineoplastic agents and TGFß were added during the labeling period.

After the labeling period the cells and medium were scraped from the wells and the wells were washed twice with 1 ml of 50 mM Tris-HCl pH 7.6 containing 25 mM ethylenediaminetetraacetic acid (EDTA), 10 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM proline. The wash solution was added to the suspension which contained cells and medium. The final suspension was freeze/thawed 3 times and the proteins were precipitated with trichloroacetic acid (TCA; final concentration 10%). The radioactive protein was separated from free [^{3}H]proline by repeated (3x) washes with 5% TCA containing 1 mM proline at 4°C.

The final sediment was dissolved in 0.75 ml 0.2 M NaOH and neutralized by the addition of 0.3 ml 1 M HEPES and 0.3 ml 0.15 M HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to determine proline incorporation into collagen 0.2 ml 20 mM Tris-HCl, pH 7.6, containing 50 mM $CaCl_2$ and 0.1 ml collagenase (chromatographically purified on a G200 gel filtration column) were added to a 0.5 ml aliquot of the solubilized sample and the mixture was incubated for 5 h at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.6 M and 3 mM, respectively. After centrifugation (10 min; 14.500 x g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyzer. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase yielded the collagen specific incorporation (collagenase-digestible protein - CDP), representing collagen synthesis. Subtraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non-collagenous protein (NCP). Incorporation into CDP and NCP is quantified per well.

The relative collagen synthesis (RCS) was calculated with the formula [17] that takes

into account the enrichment of proline in collagen compared to other proteins:

$$\% \text{ relative collagen synthesis} = \frac{CDP}{(NCP \times 5.4) + CDP} \times 100\%$$

For each experimental condition, one 6-well culture plate was used: four wells for the actual measurement of [^{3}H]proline incorporation and two wells for a cell count at the completion of the incubation period.

Differences between control and drug-treated cultures were tested for significance using a two-sided Wilcoxon test: * $p \leq 0.05$.

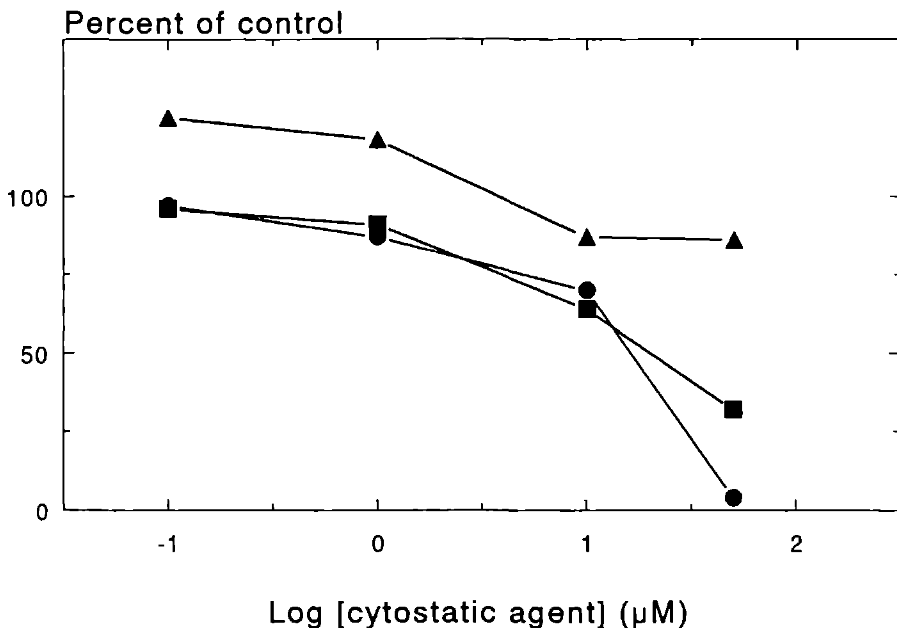


Figure 1: Effect of antineoplastic agents on fibroblast proliferation. The average of 6 measurements is given as percentage of [^{3}H]thymidine incorporation in the absence of any agent. Triangles: 5-fluorouracil; circles: cisplatin; squares: bleomycin.

RESULTS

Incubation with bleomycin or cisplatin resulted in a marked, dose-dependent inhibition of DNA synthesis in actively dividing cultures in the logarithmic growth phase (Figure 1); a 50% inhibition was noted in the concentration range of ± 17 μM. 5-Fluorouracil, even at the highest concentration used, did not reduce proliferation. In order to exclude drug effects on growth, further experiments were performed with cultures of confluent, non-dividing fibroblasts. Under these conditions, the addition of antineoplastic agents during the final 24 h of incubation did not significantly affect the number of viable cells present.

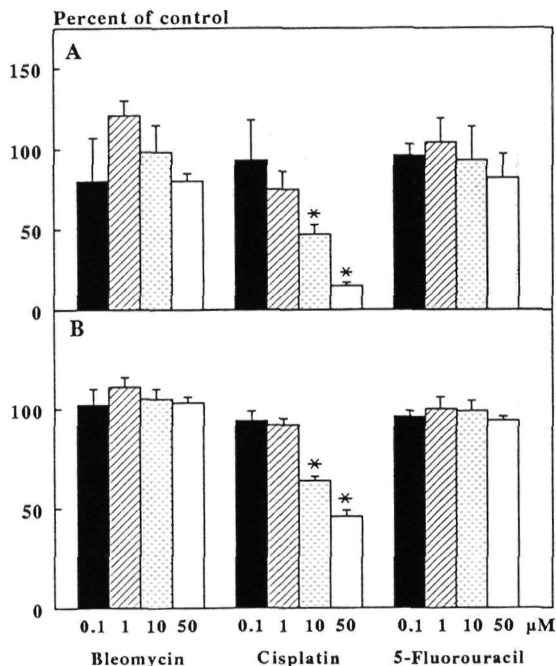
Table I: Collagen synthesis in human fibroblasts. Synthesis was measured in both cell lines cultured in the presence or absence of fetal calf serum. Results are given for both absolute (as dpm CDP/well) and relative (as %RCS) collagen synthesis. Data represent average values ± SD from five separate experiments.

	dpm CDP/well	%RCS
Skin fibroblasts		
10% serum	27299 ± 7299	3.09 ± 0.49
no serum	7194 ± 2979	1.30 ± 0.69
Colon fibroblasts		
10% serum	56730 ± 5904	2.22 ± 0.06
no serum	20842 ± 589	3.27 ± 0.44

Table I gives the average values for collagen synthesis in both fibroblast strains. Incubation under serum-free conditions reduced collagen synthesis. In skin fibroblasts the synthesis of non-collagenous protein was reduced to a lesser extent and therefore the relative collagen synthesis was also inhibited. In colon fibroblasts the opposite was true.

The effect of each compound on fibroblast collagen synthesis was examined for four concentrations (0.1, 1, 10 and 50 μM) and in cells cultured both in the absence and presence of serum. Figure 2 shows the effects on colon fibroblasts cultured in the presence of serum. Bleomycin and 5-fluorouracil did not significantly affect collagen synthesis. The higher concentrations of cisplatin strongly inhibited the incorporation of [^{3}H]proline into the CDP fraction; the fact that this inhibition was relatively specific for collagen is evident from the fact that the relative collagen synthesis was also significantly reduced, although to a somewhat lesser extent. Figure 3 depicts the

Figure 2: Effect of antineoplastic agents on collagen synthesis in colon fibroblasts. Results are given for the absolute (A) and relative (B) collagen synthesis and expressed as average value, relative to synthesis in control cultures, ± SD (4 cultures). * denotes a significant (p≤0.05, two-sided Wilcoxon test) difference between experimental and control cultures.



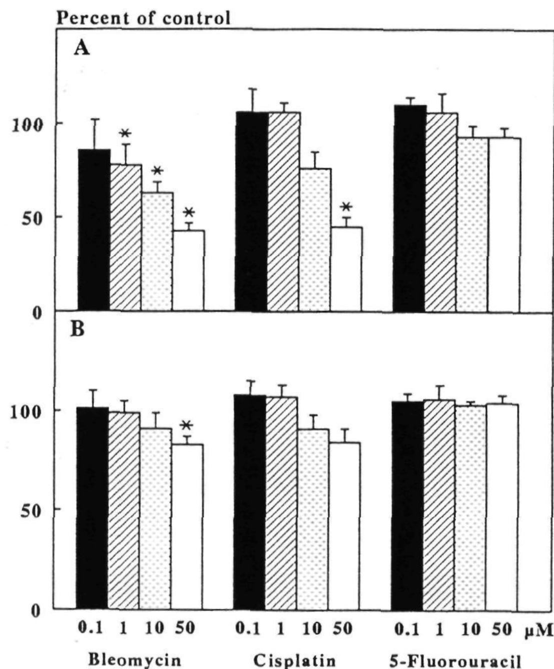


Figure 3: Effect of antineoplastic agents on collagen synthesis in skin fibroblasts. Results are given for the absolute (A) and relative (B) collagen synthesis and expressed as average value, relative to synthesis in control cultures, ± SD (4 cultures). * denotes a significant (p≤-0.05, two-sided Wilcoxon test) difference between experimental and control cultures.

effects on skin fibroblasts. Here, bleomycin inhibited the absolute collagen synthesis in a dose-dependent manner. However, this effect appeared to be hardly specific for collagen since the relative collagen synthesis was reduced significantly only at the highest concentration tested. Cisplatin induced inhibition only if added in a concentration of 50 μM but this appeared to be a general effect on protein synthesis since the relative collagen synthesis remained essentially unchanged. Again, addition of 5-fluorouracil had no effect whatsoever.

Table II: A comparison of the effects of antineoplastic agents on collagen synthesis in fibroblasts, cultured in the presence or absence of serum. Results are given for absolute (as dpm CDP/well) and relative (as %RCS) collagen synthesis. Outcome of addition of antineoplastic agents (at a 50 μM concentration) is expressed as percentile values with regard to control cultures. Data represent average values (±SD) of four cultures. *, significant ($p \leq 0.05$, two-sided Wilcoxon) difference with matching controls.

	Skin fibroblasts		Colon fibroblasts	
	10% serum	no serum	10% serum	no serum
dpm CDP/well				
control	100 ± 4	100 ± 3	100 ± 22	100 ± 20
bleomycin	43 ± 4*	64 ± 5*	80 ± 5	88 ± 31
cisplatin	45 ± 5*	43 ± 12*	15 ± 2*	59 ± 4*
5-FU	93 ± 5	111 ± 6*	82 ± 15	101 ± 16
RCS				
control	100 ± 3	100 ± 2	100 ± 5	100 ± 8
bleomycin	83 ± 4*	115 ± 9*	103 ± 3	89 ± 8
cisplatin	84 ± 7	96 ± 10	46 ± 3*	76 ± 4*
5-FU	104 ± 4	120 ± 8*	94 ± 2	103 ± 9

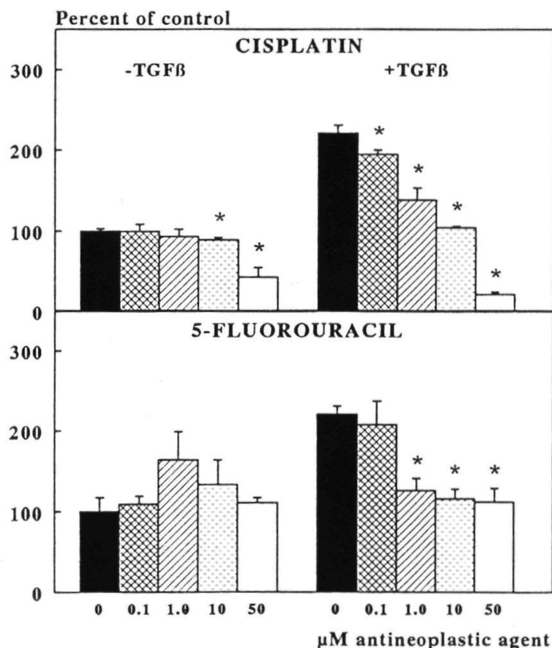
The preceding data were obtained from cultures grown in the presence of serum. If cells were grown without serum, mostly similar results were obtained. In table II the effects of the highest concentrations of antineoplastic agents used under both conditions (50 μM) are summarized. In general, effects were qualitatively similar though sometimes quantitatively different. For instance, cisplatin inhibited the absolute collagen synthesis in colon fibroblasts by 85% if cells were grown with serum and by 41% if cells were grown without serum. The only case where the different conditions resulted in opposite effects was when skin fibroblasts were incubated with bleomycin. Here, the relative collagen synthesis was reduced in cells grown with serum and increased in cells grown without serum.

TGFß promotes collagen synthesis and we have investigated if this enhanced synthetic activity would be more susceptible to inhibition by antineoplastic agents than the basal

Table III: A comparison of the effects of antineoplastic agent on collagen synthesis in fibroblasts, cultured in the presence or absence of TGFß. Cells were cultured in the absence or presence of TGFß (5ng/ml). Results are given for absolute (as dpm CDP/well) and relative (as %RCS) collagen synthesis. Outcome of addition of antineoplastic agents (at a 1μM concentration) is expressed as percentile values with regard to control cultures. Data represent average values (±SD) of four cultures. * significant ($p \leq 0.05$, two-sided Wilcoxon) differences with matching controls.

	skin fibroblasts		Colon fibroblasts	
	-TGFß	+TGFß	-TGFß	+TGFß
CDP/well				
control	100 ± 3	221 ± 10	100 ± 20	167 ± 18
bleomycin	105 ± 8	183 ± 15*	130 ± 13	164 ± 40
cisplatin	93 ± 9	138 ± 15*	90 ± 14	139 ± 26
5-FU	164 ± 35	126 ± 15*	116 ± 27	162 ± 12
RCS				
control	100 ± 2	198 ± 9	100 ± 5	126 ± 5
bleomycin	107 ± 5*	220 ± 18*	106 ± 4	130 ± 15
cisplatin	111 ± 2*	179 ± 4*	99 ± 11	119 ± 9
5-FU	138 ± 20	188 ± 28	104 ± 19	131 ± 4

Figure 4: Effect of cisplatin and 5-fluorouracil on basal and TGFß-stimulated collagen synthesis in skin fibroblasts. Results are given for the absolute collagen synthesis, measured in the absence or presence of 5 ng/ml TGFß, and expressed as average value, relative to control cultures, ± SD (4 cultures). * denotes a significant ($p \leq 0.05$, two-sided Wilcoxon test) difference between cultures plus and minus antineoplastic agent.



activity. Since TGFß stimulation is more pronounced if cells, particularly colon fibroblasts are cultured under serum-free conditions [12] we used such conditions to study the effects of antineoplastic drugs on TGFß-enhanced synthesis. Table III shows that, if no TGFß was present, neither of the drugs, added at a 1 μM concentration, significantly inhibited collagen synthesis in both cell lines. The same was true if colon fibroblasts were supplemented with TGFß for the final 24 h of culture. However, if TGFß was added to cultures of skin fibroblasts all three compounds, at a 1 μM concentration, significantly inhibited the stimulated collagen synthesis. This effect is demonstrated further in figure 4. While cisplatin only inhibited basal collagen synthesis at concentrations higher than 10 μM, TGFß-induced collagen synthesis was already inhibited significantly at a concentration of 0.1 μM. Likewise, 5-fluorouracil was without effect on basal collagen synthesis but reduced TGFß-induced collagen synthesis to basal levels from a concentration of 1 μM upwards.

DISCUSSION

The contention that antineoplastic agents, if given in the perioperative period, are a threat to uncomplicated wound healing appears to be accepted almost universally. Although relevant clinical data are rare and fail to support this concept, there exists a volume of experimental studies on the subject which indeed demonstrates the potential for such a deleterious effect. As a consequence, cancer chemotherapy has typically been delayed for several weeks after surgical excision of tumors and the feasibility of perioperative treatment remains at doubt, even as the indications for its use appear sound [18]. Therefore, research into the effects of the various antineoplastic agents on primary processes of the healing sequence is much needed in order to further assess their suitability for perioperative application.

Fibroblast collagen synthesis is crucial to the development of wound strength. Antineoplastic agents could interfere in this process in several ways. For instance, they could prevent the release of fibroblast-chemotactic mediators from platelets and macrophages (by suppressing the numbers of these cells) or inhibit fibroblast proliferation. In addition, they could interfere directly with fibroblast collagen synthesis. With regard to this last possibility, not much is known about the effects of drugs which are commonly used today, with the possible exception of adriamycin. The

detrimental effects of this compound in experimental models are severe and undisputed: it lowers wound strength and collagen accumulation [19]. Although it was shown recently that adriamycin induces decreased gene expression for type I collagen in skin wounds of rats [20], it has also been reported to reduce the synthesis of hydroxyproline in human skin fibroblasts by inhibiting prolyl hydroxylase activity [21].
No such equivocal data exist for 5-fluorouracil, which drug remains the cornerstone for chemotherapy of colorectal cancer [1]. While earlier reports show impaired healing of experimental intestinal anastomoses [22,23], more recent data fail to support a detrimental effect [7]. We have found recently that 5-fluorouracil, administered intravenously or intraperitoneally once a day during the first 3 days after operation, did not affect strength or hydroxyproline content of intestinal anastomoses [de Waard et al., manuscript in preparation]. As with all experimental studies on the effects of antineoplastic agents on wound repair, results remain difficult to compare because variations in protocol, e.g. dose and mode and time of administration of the drug. 5-Fluorouracil is one of the few drugs that has been used clinically in the perioperative period: no evidence was found for increased anastomotic leakage [24,25]. One clear result from the present experiments is that 5-fluorouracil does not directly affect collagen synthesis in colon fibroblasts, under any of the conditions tested. In addition, fibroblast proliferation appeared to be refractory to the presence of the drug. Inhibitory effects on proliferation of human fibroblasts have been reported after longer incubations with 5-fluorouracil [26]. Still, we believe that our results support the idea that it should be safe to administer 5-fluorouracil immediately after resection of a colorectal cancer.
Cisplatin, which is often used together with 5-fluorouracil as adjuvant in the treatment of gastro-intestinal tumors, is reported to impair the development of strength in rat intestinal anastomoses [27]. Although no data on wound collagen content were supplied, our results show that cisplatin can strongly and, to a large extent, specifically suppress collagen synthetic capacity in colon fibroblasts. Thus, the evidence available cautions against the peri-operative use of cisplatin.
We have also tested the effects of bleomycin since this drug was included in the mixture administered in vivo in our previous experiments with rat intestinal anastomoses [5,6,10]. No experiments have been reported on the effects of bleomycin

alone on intestinal healing. Collagen synthesis in colon fibroblasts remains unaffected by bleomycin, in contrast to skin fibroblasts where bleomycin induced a significant, though not very specific, inhibition. This appears in agreement with earlier results which show that bleomycin, at concentrations around 1 μM, though increasing the amount of procollagen mRNA in the cell layer [28], eventually induces an inhibition of the synthesis of collagenous protein in the medium because it is being rapidly degraded intracellularly and extracellularly [29]. It should be emphasized that the method employed in our experiments measures the net accumulation of collagen in cell layer plus medium, the bulk of the collagen being present in the medium.

The latter results illustrate the differences observed by us between colon and skin fibroblasts. Both absolute and relative collagen synthesis in colon fibroblasts remain unaffected by bleomycin while being inhibited in skin fibroblasts. Cisplatin strongly supresses absolute and relative collagen synthesis in colon cells, while only affecting the absolute synthesis in skin fibroblasts at the highest concentration used. These results further extend our recent findings [12] that fibroblasts from both tissues exhibit divergent reactions to various stimuli and therefore may cause wounds in skin and intestine to behave differently under certain conditions.

It is becoming increasingly clear that TGFß plays an important regulatory role in wound healing [15,16]. It has been shown that the expression of mRNA for TGFß is decreased in adriamycin-impaired skin wounds [20] and that exogenous TGFß reverses the adriamycin-induced inhibition of collagen accumulation in wound chambers [30]. Therfore, it is interesting to observe that, if the collagen synthesis in skin fibroblasts is stimulated by addition of TGFß to the cultures, the additional activity is significantly inhibited by concentrations of antineoplastic agents which are much lower than those necessary to inhibit the basal synthetic activity. Possibly, this effect of antineoplastic agents on TGFß-elicited synthetic activity has far more direct implications for the process of wound healing than their effects on basal fibroblast activity. The fact that TGFß-stimulated collagen synthesis in colon fibroblasts remains unaffected by 5-fluorouracil would then give additional weight to the argument that this drug is relatively harmless to colonic wound healing.

Altogether, the present data show that antineoplastic agents can have diverse effects on fibroblast collagen synthesis. If one wants to assess possible effects of a drug on

tissue wound repair by measuring its effects on fibroblast collagen synthesis, it appears indicated to use fibroblasts derived from that particular tissue and to measure also after stimulation by TGFß.

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CHAPTER 7

COLLAGEN SYNTHESIS IN FIBROBLASTS FROM HUMAN COLON: REGULATORY ASPECTS AND DIFFERENCES WITH SKIN FIBROBLASTS

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Gut (1992), 33,

ABSTRACT:

The purpose of this study is two-fold: (1) to examine regulation of collagen synthesis in human colon fibroblasts and (2) to compare the results from colon fibroblasts with those obtained in fibroblasts from human skin. We investigated the effects of interleukin-1ß, tumor necrosis factor-α, interferon-gamma, transforming growth factor-ß, dexamethasone and the calcium ionophore A23187. All compounds were tested both in the absence and in the presence of fetal calf serum in the culture medium.

The process of collagen synthesis in fibroblasts from colon and skin appears to be affected differently by these regulatory compounds. The most pronounced differences were that

- the relative collagen synthesis increases in dermal fibroblasts and decreases in colon fibroblasts upon addition of serum;
- in the presence of serum, interleukin-1ß inhibits collagen synthesis in skin fibroblasts but not in colon fibroblasts;
- dexamethasone suppresses the relative collagen synthesis in skin fibroblasts but not in colon fibroblasts;
- transforming growth factor-ß stimulates the collagen synthesis in dermal fibroblasts in the presence of serum, but inhibits the process in colon fibroblasts.

Since fibroblasts are the primary sources of collagen needed during wound repair, these results may offer (part of) the explanation why wounds in skin and intestine appear to behave differently under certain conditions.

INTRODUCTION:

Wound healing is defined as a highly regulated sequence of various cellular events leading to reconstitution of tissue integrity following injury [1]. Fibroblasts play an important role in this sequence by producing extracellular matrix components such as the different types of collagen and fibronectin [2-4]. Collagen is particularly important for the development of wound strength. In skin wounds, the increase in hydroxyproline parallels the increase in tensile strength [5]. Also, a direct relationship is observed between collagen diameter and tensile strength [6]. Collagen metabolism

is closely controlled during the process of wound repair. Regulation is probably achieved both by the interaction of fibroblasts with the surrounding extracellular matrix as well as by cytokines and growth factors known to regulate specifically collagen gene expression and collagen metabolism [7-9].

Most studies on wound healing have used skin, mainly because of the easy accessibility of this tissue. Although one would certainly like to think that all wounds heal by a single common mechanism, various authors [10-12] have expressed the necessity to exert caution in extrapolating the results from research on skin repair to the healing of other soft tissues like intestine. Anastomotic repair in the intestine has been a topic of study in our laboratory for some time [13]. We have indeed found indications that intestinal wounds and skin wounds react differently to in vivo administration of various drugs. For instance, administration of methylprednisolone does not affect strength or collagen content of intestinal anastomoses while impairing healing in skin [14]. Also, administration of D-penicillamine, a lathyrogen which inhibits collagen cross-linking, leaves anastomotic strength and collagen solubility unaffected while it lowers strength and increases collagen solubility in skin wounds [15]. Although various explanations for these phenomena are possible, the question arises if production and metabolism of collagen in wounds of intestine and skin are under different control.

Since fibroblasts are the primary collagen-producing cells in healing wounds, we decided to study the regulation of the collagen production rate in fibroblasts from human colon. Although recent work describes enhanced collagen gene expression in 'fibroblast-like' cells in experimental colonic anastomoses [16], no quantitative data are available as yet regarding collagen synthesis in colon fibroblasts. We have compared the results with those obtained with fibroblasts from human skin. The data show that synthesis of collagenous protein in cells from both tissues is affected differently by various regulatory compounds.

MATERIALS AND METHODS:

Materials:

All supplies for cell culture were purchased from Life Technologies (Breda, The Netherlands). Transforming growth factor-ß (TGFß) from bovine bone was a gift

from dr. G. Ksander (Celtrix Labs, Palo Alto, USA). Interferon-gamma (IFNgamma) was obtained from Boehringer (Ingelheim, Germany). Both human recombinant interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNFα) were a gift of prof. dr. J.W.M. van der Meer (University Hospital Nijmegen). L-[2,3-^{3}H]Proline (1,63 Tbq/mmol) was purchased from Amersham International, England. Collagenase (type VII), calcium ionophore (A23187) and dexamethasone were obtained from Sigma (St. Louis, USA). All other reagents were of analytical grade (Merck, Darmstadt, Germany).

Cell culture:

Normal human colon fibroblasts (HCF) were obtained from the American Type Culture Collection (CRL-1459). Human skin fibroblasts (HSF) were obtained from explants of skin biopsies of a healthy adult. Both the HSF and HCF were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with antibiotics (100 UI/ml penicillin and 100 μg/ml streptomycin) and 10% fetal calf serum (FCS) at 37°C in a 5% CO_2, 95% air humidified atmosphere. Cells were used between the third and tenth passage.

Assay of fibroblasts collagen production:

Assay of collagen production by steady state, visually confluent fibroblasts was assessed over a 24-h period by [^{3}H]proline incorporation into collagenous protein.

Freshly trypsinized fibroblasts were plated in 6 wells plates at a density of approximately 1.5×10^5 cells/well in 2 ml DMEM plus 10% FCS. Three days after plating the medium was removed and replaced by the same medium or with DMEM without serum. In the latter case the wells were first washed twice with phosphate buffered saline (PBS). Twenty four hours later the medium was replaced by the same medium plus ascorbic acid (50 μg/ml), ß-aminopropionitrile (50 μg/ml) and 2 μCi/ml [2,3-^{3}H]proline for the final 24 h of culture. IL-1ß, TNFα, IFNgamma, TGFß, dexamethasone or calcium-ionophore A23187 were added during the labeling period. However, IL-1ß and TNFα were also added during the 24 h culture period prior to the labeling period. If the calcium ionophore was given, 1.5 mM $CaCl_2$ was added to the culture medium simultaneously.

After the labeling period the cells and medium were scraped from the wells and the wells were washed twice with 1 ml of 50 mM Tris-HCl pH 7.6 containing 25

mM ethylenediaminetetraacetic acid (EDTA), 10 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM proline. The wash solution was added to the suspension which contained cells and medium. The final suspension was freeze/thawed 3 times and the proteins were precipitated with trichloroacetic acid (TCA; final concentration 10%). The radioactive protein was separated from free [^{3}H]proline by repeated (3x) washes with 5% TCA containing 1 mM proline at 4°C.

The final sediment was dissolved in 0.75 ml 0.2 M NaOH and neutralized by the addition of 0.3 ml 1 M HEPES and 0.3 ml 0.15 M HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to determine proline incorporation into collagen 0.2 ml 20 mM Tris-HCl, pH 7.6, containing 50 mM $CaCl_2$ and 0.1 ml collagenase (chromatographically purified on a G200 gel filtration column) were added to a 0.5 ml aliquot of the solubilized sample and the mixture was incubated for 5 h at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.6 M and 3 mM, respectively. After centrifugation (10 min; 14.500 x g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyzer. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase yielded the collagen specific incorporation, which will be referred to as collagenase-digestible protein (CDP). Subtraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non-collagenous protein (NCP). Incorporation into CDP and NCP is quantified per well.

The relative collagen synthesis was calculated with the formula [17] that takes into account the enrichment of proline in collagen compared to other proteins:

$$\%\ \text{relative collagen synthesis} = \frac{\text{CDP}}{(\text{NCP} \times 5.4) + \text{CDP}} \times 100\%$$

RESULTS:

Collagen synthesis in the two fibroblast strains was always measured both in the presence and in the absence of 10% serum. In the presence of serum, HCF produce more collagen and non-collagenous protein than HSF (table 1). This

difference is maintained if the incorporation of label into the fractions is expressed per cell (results not shown). If serum is omitted, synthetic capacity decreases. The absolute collagen synthesis is lowered by 63 and 74% in HCF and HSF, respectively. However, the synthesis of non-collagenous protein is affected far more strongly in HCF. As a result, serum-free conditions cause the relative collagen synthesis to be more than halved in HSF and to be increased by nearly 50% in HCF.

Table 1: Effect of serum on collagen and non-collagenous protein synthesis. Synthesis was measured in both cell lines cultured in the presence and absence of fetal calf serum. Results represent average values ± SD from five separate experiments.

	HSF		HCF	
	10 % serum	0 % serum	10 %	0 %
dpm CDP/well	27299 ± 7299	7194 ± 2979	56730 ± 5904	20842 ± 589
dpm NCP/well	164301 ± 34204	114442 ± 52967	464064 ± 40956	117774 ± 33346
%RCS	3.09 ± 0.49	1.30 ± 0.69	2.22 ± 0.06	3.27 ± 0.44

Table 2: Effect of IL-1ß on collagen and non-collagenous protein synthesis. Both colon fibroblasts (HCF) and skin fibroblasts (HSF) were grown in 10% serum or serum-free culture medium with different concentrations of IL-1ß. Results are expressed as percentile values with regard to the control cultures without IL-1ß. Data represent average values (±SD) of four cultures. Differences between control and cytokine-treated cultures were tested for significance using a two-sided Wilcoxon test: * p ≤ 0.05.

		HSF		HCF	
	IL-1ß units/ml	10% serum	0% serum	10% serum	0% serum
CDP/well					
	0	100 ± 14	100 ± 6	100 ± 10	100 ± 6
	1	77 ± 14	64 ± 10*	108 ± 19	103 ± 14
	50	69 ± 2*	50 ± 9 *	104 ± 8	49 ± 5*
NCP/well					
	0	100 ± 13	100 ± 6	100 ± 9	100 ± 12
	1	98 ± 12	98 ± 11	106 ± 12	112 ± 5
	50	98 ± 11	83 ± 11	126 ± 6 *	90 ± 10
%RCS					
	0	100 ± 11	100 ± 3	100 ± 3	100 ± 5
	1	78 ± 4 *	67 ± 4 *	102 ± 8	92 ± 12
	50	67 ± 4 *	62 ± 4 *	83 ± 3 *	56 ± 12*

Cytokines are potential regulators of protein synthesis. In HCF cultured with serum the absolute collagen synthesis is not affected by IL-1ß (table 2). Since the

synthesis of NCP increases with the higher concentration of IL-1ß used, the relative collagen synthesis is lowered. In the absence of serum, both absolute and relative collagen synthesis are inhibited by 50 units/ml IL-1ß. The collagen synthesis in skin fibroblasts appears far more susceptible to the cytokine; the lower concentration suppresses synthesis independent of the presence of serum. The bulk of non-collagenous protein synthesis remains unaffected by the addition of IL-1ß.

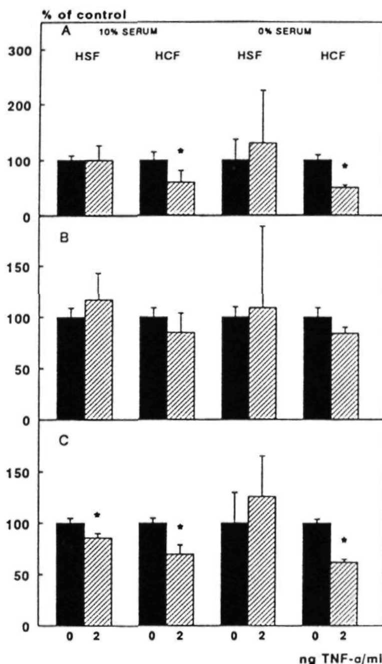
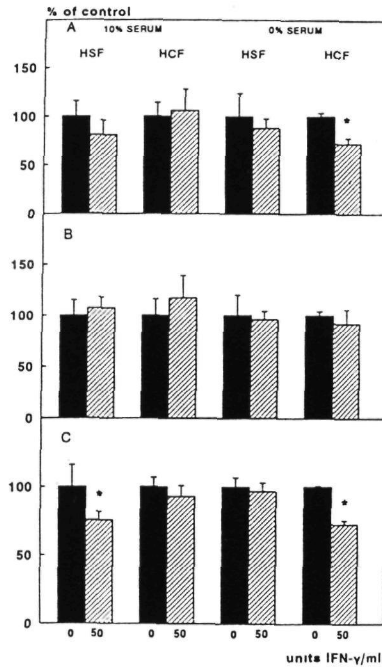


Figure 1. Effect of TNFα on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines, were incubated for 48 h with or without TNFα, both in the presence or absence of FCS. Results are expressed as percentage of control without added TNFα. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (±SD) of four cultures is given. Differences between control and cytokine treated cell cultures are tested for significance using a two-sided Wilcoxon test: * $p \leq 0.05$.

TNFα, at a concentration of 2 ng/ml, does not affect the synthesis of non-collagenous protein in either of the fibroblast strains (figure 1). If the skin fibroblasts are cultured without serum, no effect on collagen synthesis is observed. In the presence of serum, the relative collagen synthesis is reduced by 14%. The potentially inhibitory effect of TNFα is more explicit in the HCF under both culture conditions. For instance, the absolute collagen synthesis is reduced by 50% if cells are grown without added serum. At lower concentrations, 0.02 and 0.2 ng/ml, TNFα induces no alterations in synthetic activity (results not shown).

Figure 2. Effect of IFNgamma on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines, were incubated for 24 h with or without IFNgamma, both in the presence or absence of FCS. Results are expressed as percentage of control without added IFNgamma. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (±SD) of four cultures is given. Differences between control and cytokine treated cell cultures are tested for significance using a two-sided Wilcoxon test: * $p \leq 0.05$.



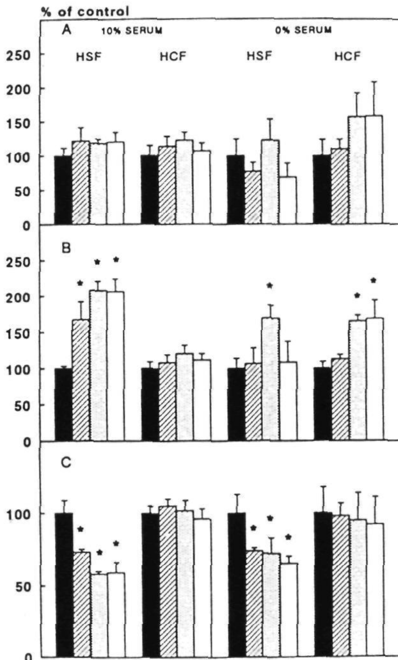


Figure 3. Effect of dexamethasone on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines, were incubated for 24 h with or without dexamethasone, both in the presence or absence of FCS. Results are expressed as percentage of control without added dexamethasone: black bars represent controls, striped bars 10^{-9} M, dotted bars 10^{-7} M and open bars 10^{-5} M dexamethasone. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (±SD) of four cultures is given. Differences between control and dexamethasone treated cell cultures are tested for significance using a two-sided Wilcoxon test: * $p \leq 0.05$.

Both cell lines react essentially similar to the addition of IFNgamma to the culture medium. Again, total protein synthesis remains unaffected, while collagen synthesis is lowered (figure 2). However, in HSF the decrease is more apparent in the presence of serum while in HCF significantly reduced CDP and %RCS values are only observed after culture without serum.

The synthetic activity in HCF is hardly affected by the glucocorticoid dexamethasone (figure 3): in the presence of serum neither the collagen synthesis nor the synthesis of non-collagenous protein changes after addition of the drug. Both activities appear to be stimulated in serum-free cultures, at least at the higher concentrations of dexamethasone. As a result, the relative collagen synthesis remains unaffected. HSF react differently: in the presence of serum the synthesis of non-collagenous protein is doubled by dexamethasone while the absolute collagen synthesis is not influenced. Thus, the relative collagen synthesis decreases significantly, from concentrations of 10^{-9} M dexamethasone upwards.

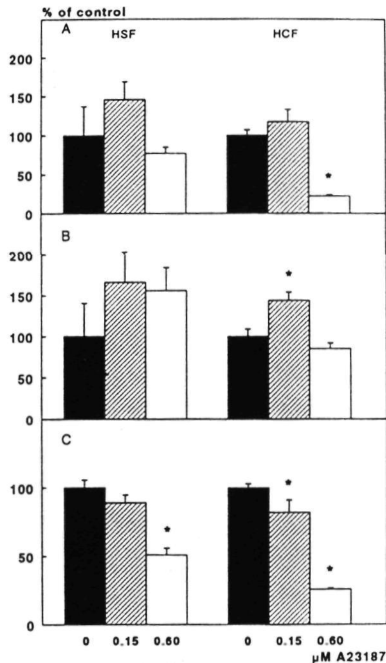
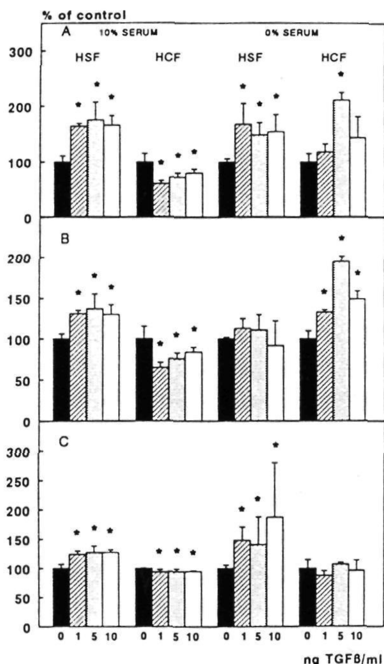


Figure 4. Effect of calcium ionophore A23187 on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines, were incubated for 24 h with or without A23187, in absence of FCS. Results are expressed as percentage of control without added A23187. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value (±SD) of four cultures is given. Differences between control and ionophore treated cell cultures are tested for significance using a two-sided Wilcoxon test: * $p \leq 0.05$.

Another way to regulate collagen synthesis in colon fibroblasts is the addition of the calcium ionophore A23187. While no effects are observed in the presence of serum (results not shown), culturing cells in the presence of 0.6 μM A23187 suppresses the collagen synthesis by more than 75% (figure 4). Although HSF are also found to be susceptible to the presence of the ionophore the picture seems somewhat different: the significant decrease in the relative collagen synthesis appears more a result from an increase in synthesis of non-collagenous protein than from a lowered absolute collagen synthesis.

Figure 5. Effect of TGFß on collagen and non-collagen protein synthesis. Visually confluent fibroblasts from both cell lines, were incubated for 24 h with or without TGFß, both in the presence or absence of FCS. Results are expressed as percentage of control without added TGFß. Values represent absolute collagen synthesis (A), synthesis of non-collagenous protein (B) or the relative collagen synthesis (C). The average value ($\pm$SD) of four cultures is given. Differences between control and growth factor treated cell cultures are tested for significance using a two-sided Wilcoxon test: * $p \leq 0.05$.



Growth factors, and especially TGFß, are thought to stimulate fibroblast collagen synthesis. This is indeed the case in HSF, regardless of culture conditions (figure 5). In both cases, the absolute and relative collagen synthesis are increased, although the latter less so in 10% serum since here the synthesis of non-collagenous protein is also significantly enhanced. HCF react just the opposite, at least in the

presence of serum. Both collagen and non-collagenous protein synthesis are suppressed significantly. In the absence of serum, collagen synthesis in HCF is enhanced by TGFß, particularly at a concentration of 5 ng/ml. However, the synthesis of other proteins is stimulated to the same extent and thus the relative collagen synthesis remains unchanged.

Finally, we investigated if TGFß could negate the inhibitory effects of IFNgamma and A23187 on collagen synthesis in colon fibroblasts cultured under serum-free conditions. As shown before, both IFNgamma and A23187 significantly suppress collagen synthesis, absolute and relative, while TGFß stimulates absolute collagen synthesis. TGFß is able to overcome the negative effects of IFNgamma (table 3): if added simultaneously, values for the parameters observed are similar to those observed in the presence of TGFß alone. In contrast, TGFß cannot restore the inhibition induced by A23187. Culture in the presence of both compounds results in a massive suppression of collagen synthesis, similar to that induced by A23187 alone.

Table 3: Collagen synthesis in HCF under serum-free conditions. Cells were grown in serum-free medium. Results are expressed as percentile values with regard to control cultures. Data represent average values (±SD) of four cultures. Differences between control and other cultures were tested for significance using a two-sided Wilcoxon test: * $p \leq 0.05$.

	CDP/well	NCP/well	%RCS
control	100 ± 13	100 ± 13	100 ± 11
+ TGFß (10 ng/ml)	140 ± 10*	146 ± 4 *	95 ± 5
+ IFNgamma (1000 u/ml)	61 ± 5 *	82 ± 6	76 ± 9 *
+ IFNgamma + TGFß	152 ± 8 *	153 ± 9 *	99 ± 5
+ A23187 (0.6 μM)	14 ± 2 *	71 ± 7 *	20 ± 1 *
+ A23187 + TGFß	16 ± 2 *	79 ± 3 *	21 ± 2 *

DISCUSSION:

Collagen synthesis is an essential and universal feature of wound repair. After construction of an anastomosis in the intestine the synthetic capacity is strongly, and specifically, elevated in the wound area [18,19]. Since collagen synthesis is thought to be crucial to the development of anastomotic strength, the study of its regulation is of great potential interest. Leakage of colonic anastomoses is a phenomenon that occurs rather frequently [20] and insight into regulation of the processes which are essential in the repair sequence could contribute to the development of measures to avoid this

complication. The cells most likely to be responsible for collagen synthesis are the fibroblasts which migrate into the wound and display great proliferative activity. Thus, we investigated collagen synthesis in an established line from human colon fibroblasts [21] and compared the results with those obtained with fibroblasts from human skin. In the literature on fibroblast collagen synthesis discrepancies between studies are often explained by supposedly different culture conditions, particularly with respect to the presence of serum [22]. In order to discover if such variations in culture conditions indeed change the effects of the regulatory compounds studied, synthetic activity was measured both in the presence and absence of serum.

It has been shown that addition of serum to fibroblast cultures stimulates collagen synthesis [23], probably at the transcriptional level [24]. Although colon fibroblasts behave similarly in this respect they seem to be unique in the sense that the synthesis of non-collagenous protein is stimulated to a greater degree, resulting in a lower relative collagen synthesis under serum-supplemented conditions.

IL-1ß inhibits collagen synthesis in colon fibroblasts cultured in the absence of serum. We found silmilar results for skin fibroblasts, which observation is in agreement with those reported by others [22,25]. The fact that such inhibition was not seen in the presence of serum confirms a similar observation by Duncan and Berman [26], although our skin fibroblasts are also responsive under these conditions and display a suppressed collagen synthesis.

TNFα inhibits the collagen synthesis in colon fibroblasts. A similar effect with comparable TNF concentrations has been reported for skin fibroblasts [27,28], while our own results show that only the relative collagen synthesis decreases in skin fibroblasts cultured in the presence of serum. These data contradict those of others [26] who show an increased collagen synthesis in dermal fibroblasts cultured without serum.

Studies on IFNgamma show that this compound universally inhibits collagen synthesis in a variety of target cells, e.g. fibroblasts [27,29] and fetal bone cultures [30]. In this respect, colon fibroblasts are no exception although significant inhibition is only measured under serum-free conditions.

Neither the absolute nor the relative collagen synthesis in colon fibroblasts are significantly affected by dexamethasone. Most studies with skin fibroblasts indicate

that glucocorticoids reduce collagen synthesis [31]: decreased levels of mRNA suggest pretranslational regulation [32]. In the present study, a reduction in the absolute collagen synthesis is only apparent, and not significantly so, if dermal fibroblasts are grown without serum. The decreased relative collagen synthesis is, certainly in serum-supplemented cultures, the result of a stimulation in the synthesis of non-collagenous protein. In animal studies we have found that colonic wound healing is unaffected by administration of corticosteroids at doses which impair dermal wound healing [14]. Therefore, it is interesting to observe that collagen synthesis in fibroblasts from colon seems refractory to corticosteroids. Possibly, a difference in regulation of this fibroblast function may (partly) explain the divergent healing patterns in both tissues.

Growth factors, and especially TGFß, are known to stimulate collagen synthesis in fibroblasts from a variety of tissues [33-35]. Our results with dermal fibroblasts confirm earlier results reported for cells from this tissue. In contrast, synthetic activity in human colon fibroblasts is significantly inhibited in the presence of serum. While there has been a report on negative effects of TGFß on collagen synthesis in hepatocytes [36], this is the first evidence that TGFß may inhibit collagen synthesis in fibroblasts. Fukamizu and Grinell [37] described decreased collagen synthesis in long-term fibroblast cultures, possibly as the result of lowered fibroblast activity concomitant with spatial reorganization of the extracellular matrix. In the absence of serum, absolute collagen synthesis in colon fibroblasts is indeed stimulated by TGFß in a concentration of 5 ng/ml. At this concentration, TGFß can overcome the inhibition induced by IFNgamma under these conditions, suggesting that both cytokines affect collagen synthesis through the same regulatory pathway. Simultaneous exposure of either skin fibroblasts [38] or gingival fibroblasts [39] to both IFNgamma and TGFß did reverse the stimulation in collagen production observed with TGFß alone, suggesting the presence of independent mechanisms.

Increasing the intracellular calcium concentration by addition of the ionophore A23187 together with extra calcium to the culture medium strongly suppresses collagen synthesis in colon fibroblasts. If the ionophore is added together with TGFß, collagen synthesis remains inhibited to the same extent. Thus it seems that the regulatory action of TGFß is dependent upon a low intracellular calcium level.

Next to investigating the behavior of collagen synthesis in colon fibroblasts under addition of various regulatory compounds, the aim of this study was to establish if fibroblasts from colon and skin behave differently in this respect. This indeed appears to be the case:

- The relative collagen synthesis increases in dermal fibroblasts and decreases in colon fibroblasts upon the addition of serum.
- In the presence of serum IL-1ß inhibits collagen synthesis in skin fibroblasts but not in colon fibroblasts.
- Dexamethasone suppresses the relative collagen synthesis in skin fibroblasts but not in colon fibroblasts.
- TGFß stimulates the collagen synthesis in dermal fibroblasts cultured in the presence of serum but inhibits the process in colon fibroblasts.

The other differences observed are less explicit and merely a matter of degree and fall within the variations observed by us (results not shown) and others [26] for different cell lines from the same tissue. However, those mentioned above, and most particularly the discrepancy measured in the presence of TGFß, suggest that repair activities of fibroblasts in colon and skin may be under different control. Wound fibroblasts obtained from implanted sponges differ from normal dermal fibroblasts with regard to their capacity to synthesize collagen and remodel collagen lattices [40]. Thus, the wound environment may alter fibroblast phenotype. Our results indicate that fibroblasts from skin and colon may exhibit divergent reactions to cytokines or growth factors produced during the inflammatory part of the healing sequence and therefore may cause wounds in skin and intestine to behave differently under certain conditions.

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SUMMARY

Despite great advances in gastrointestinal surgery, particularly with respect to techniques and materials, the occurrence of anastomotic dehiscence remains a serious problem after operations in the large bowel. The reported incidence of leakage of large-bowel anastomoses varies strongly between clinics but ranges on average between 4 and 13%. This percentage is considerably lower after surgery in the small bowel. Anastomotic leakage which causes clinical symptoms leads to serious morbidity, lengthened hospital stay and a mortality of approximately 30%. Research into the mechanisms underlying - disturbed - wound healing in the intestine is thus essential in the search for measures which may prevent anastomotic leakage. Since the incidence of this phenomenon is clearly different for small and large bowel, comparison of the process in both intestinal segments may supply valuable information.

The strength of the intestinal wall lies within the submucosa; this connective tissue layer consists predominantly (>80%) of collagen fibrils. After resection and restoration of continuity early anastomotic strength will depend on the integrity of the existing connective tissue which must anchor the sutures. Thereafter newly-synthesized collagen fibrils, which bridge the tissue defect, will determine anastomotic strength and restore pre-operative strength to the bowel wall. Thus, collagen synthesis is a crucial process within the wound repair sequence and as such forms the subject of study in this thesis. **Chapter 1** briefly describes structure and synthesis of collagen and the importance of this connective tissue protein for the strength and integrity of the intestinal wall.

Chapter 2 describes the system used to measure *in vitro* collagen synthesis in explants of rat intestine. Tissue segments are finely minced and shortly (3 h) incubated with [^{3}H]proline. Thereafter, the incorporation of radioactivity into total protein and collagenase-digestible protein is measured. This allows the determination of both absolute and relative (as a percentage of non-collagenous protein synthesis) collagen synthesis. Collagen synthesis in the large bowel (colon) is significantly higher than in the small bowel (ileum), consisting 0.37 and 0.21%, respectively, of total protein synthesis. Addition of non-labeled proline during incubation strongly increases synthesis of both collagen and non-collagenous proteins, suggesting that proline

transport is rate-limiting. Collagen synthesis in colon and ileum significantly decreases with age. Such an effect is expected on the basis of literature on protein synthesis during aging and suggests that changes which occur in the intestinal wall in vivo, can be assayed with this in vitro measurement.

This method is used to follow collagen synthesis in intestinal anastomoses in the rat (**chapter 3**). In order to compare processes in small and large bowel an anastomosis is constructed in both ileum and colon of the same animal. The rats are killed 3 or 12 hours, or 1, 2, 3, 4, 7 or 28 days after operation. In the ileum collagen synthesis is significantly enhanced already 3 hours postoperatively and remains strongly stimulated during the first week with a maximal, ten-fold, increase after 4 days. Stimulation of the collagen synthetic capacity in colon is delayed compared to that in ileum; here, maximal increase is six-fold. The postoperative stimulation of collagen synthesis is higher in ileum than in colon of almost all (48) animals. These results demonstrate that the ileum responds more quickly and strongly to wounding than the colon, at least as far as the production of new collagen is concerned.

In the experiment described above two anastomoses have been constructed in each animal in order to allow a direct comparison between ileal and colonic healing. We have also investigated if, and to what extent, construction of an anastomosis in either ileum or colon would affect collagen synthesis throughout the uninjured bowel wall (**chapter 4**). For this purpose, synthesis has been measured, both one and four days after operation, on 10 locations outside the immediate anastomotic area, six in the small bowel and four in the large bowel. Results have been compared with data from non-operated controls. Collagen synthesis in the intestine of control rats is relatively uniform throughout the large bowel; in the small bowel synthesis is significantly higher in the most proximal and distal segments than in the tissue in between. Postoperative stimulation of collagen synthesis is not restricted to the anastomotic (5 mm) segment but also occurs at more distant sites. This phenomenon is, particularly in the small bowel, to some extent specific for collagen because stimulation of non-collagenous protein synthesis is considerably less pronounced. Anastomotic construction in the colon only marginally affects collagen synthesis in the ileum and vice versa. Thus, if two anastomoses are constructed within one animal (as is the case in the experiments described in chapter 3), the effect of one anastomosis on collagen synthesis in the

other is negligible.

It is generally assumed that patients which receive cytostatic agents in the peri-operative period will run an increased risk for impaired wound healing and thus anastomotic leakage. Earlier experiments in our laboratory have shown a considerable loss of anastomotic strength in the first week after operation if rats receive a combination of bleomycin, 5-fluorouracil and cisplatin daily for 5 consecutive days and are operated on the third day of this course. **Chapter 5** describes the measurement of anastomotic collagen synthesis at 3 and 7 days after operation in rats which are subjected to such a regimen: cytostatics are administered either intravenously or intraperitoneally. This treatment results in a massive and specific inhibition of anastomotic collagen synthesis at 3 days postoperatively, both in ileum and in colon. Administration via the intraperitoneal route is more detrimental in this respect than administration via the intravenous route. If cytostatics are injected intravenously, collagen synthesis is back to the level of the controls after 7 days; at this time synthesis is still inhibited in the animals which received the drugs intraperitoneally. It is concluded that anastomotic loss of strength is caused by cytostatics-induced inhibition of collagen synthesis.

The intestinal wall contains various types of cells which are capable of synthesizing collagen: fibroblasts, smooth muscle cells, endothelial cells and even macrophages. It stands to reason that fibroblasts, which migrate into the wound area, are mainly responsible for collagen synthesis during tissue repair. The next two chapters describe experiments which concern the regulation of collagen synthesis in human fibroblasts. There exists evidence that intestinal wounds may react differently to cutaneous wounds, e.g. after systemic administration of certain drugs. Therefore, a comparison is made between collagen synthesis in colon and skin fibroblasts.

Negative effects of cytostatic drugs, as described in the preceding chapter, may be the result of diminished fibroblast migration and/or proliferation. However, it appears that various compounds also directly, and specifically, inhibit fibroblast collagen synthesis (**chapter 6**). Synthesis in colon fibroblasts is strongly inhibited by cisplatin (in concentrations higher than 10 μM) but not by similar molar concentrations (between 0.1 and 50 μM) bleomycin or 5-fluorouracil. Collagen synthesis in fibroblasts from human skin is inhibited, dose-dependently, by bleomycin but not by cisplatin or 5-

fluorouracil. If collagen synthesis is stimulated by the addition of transforming growth factor-ß (TGFß), the cytostatic drugs inhibit the additional synthesis at far lower concentrations than those needed to suppress basal synthesis. In this case collagen synthesis in skin fibroblasts, but not in colon fibroblasts, is also inhibited by 5-fluorouracil. These findings seems very relevant since TGFß is increasingly expressed during wound healing. Thus, delayed healing during administration of cytostatics may very well be caused by a direct inhibition of fibroblast collagen synthesis. In addition, it seems that fibroblasts from skin and colon may behave very differently.

This last finding is further elaborated in **chapter 7**: a study is made into the divergent effects of a number of regulatory compounds on collagen synthesis in fibroblasts from colon and skin. It concerns the effects of serum, interleukin-1ß (IL-1ß), tumor necrosis factor, interferon-gamma, TGFß, dexamethasone and the calcium ionophore A23187. All assays are performed both in the absence and in the presence of fetal calf serum in the culture medium. The most pronounced differences between colon and skin fibroblasts are that

- the relative collagen synthesis increases in dermal fibroblasts and decreases in colon fibroblasts upon addition of serum;
- in the presence of serum, IL-1ß inhibits collagen synthesis in skin fibroblasts but not in colon fibroblasts;
- dexamethasone suppresses the relative collagen synthesis in skin fibroblasts but not in colon fibroblasts;
- TGFß stimulates the collagen synthesis in dermal fibroblasts in the presence of serum but inhibits the process in colon fibroblasts.

These results may offer (part of) the explanation why wounds in skin and intestine appear to behave differently under certain conditions.

SAMENVATTING

Ondanks grote vorderingen op het gebied van de darmchirurgie, met name wat betreft technieken en gebruikte materialen, blijft het optreden van naadlekkage een groot probleem bij operaties aan de dikke darm. De frequentie van lekkage van naden in de dikke darm kan sterk variëren van kliniek tot kliniek maar ligt in het algemeen waarschijnlijk tussen de 4 en 13%. Dit percentage ligt aanzienlijk hoger dan dat voor naden in de dunne darm. Indien naadlekkage klinische symptomen veroorzaakt leiden de complicaties tot een sterk verlengd verblijf in het ziekenhuis en een mortaliteit van ongeveer 30%. Bestudering van de mechanismen welke ten grondslag liggen aan de wondgenezing in de darm is daarom van groot belang bij het zoeken naar aangrijpingspunten ter voorkoming van naadlekkage. Omdat de frequentie waarmee deze complicatie optreedt zo duidelijk verschilt tussen dunne en dikke darm, kan vergelijking van processen in beide darmsegmenten hierbij waardevolle informatie verschaffen.

De sterkte van de darmwand wordt bepaald door de submucosa; deze bindweefsellaag bestaat voornamelijk (>80%) uit collageenfibrillen. Na resectie van een darmsegment en herstel van de continuïteit zal de sterkte van de aangelegde naad in eerste instantie afhankelijk zijn van de sterkte van het weefsel waarin de hechtingen zijn verankerd. Na enkele dagen zal de sterkte van de naad steeds meer worden bepaald door nieuwe collageenfibrillen die het weefseldefect overbruggen en de pre-operatieve sterkte van de darmwand herstellen. Derhalve is de collageensynthese een cruciaal onderdeel binnen het wondgenezingsproces en vormt als zodanig het onderwerp van studie in dit proefschrift. In **hoofdstuk 1** wordt een beknopte samenvatting gegeven van structuur en synthese van collageen en van het belang van dit structurele eiwit voor de sterkte van de darmwand.

Hoofdstuk 2 bevat een beschrijving van het gebruikte systeem om in vitro collageensynthese te meten in explantaten van rattedarm. Weefselsegmenten worden fijngeknipt en kortdurend (3 uur) geincubeerd met [^{3}H]proline. Vervolgens wordt de incorporatie van radioactiviteit in totaal eiwit en collagenase-afbreekbaar eiwit bepaald. Hieruit kunnen zowel de absolute als de relatieve (als percentage van de synthese van niet-collagene eiwitten) collageensynthese worden bepaald. De

collageensynthese is significant hoger in de dikke darm (colon) dan in de dunne darm (ileum) en bedraagt, respectievelijk, 0.37 en 0.21% van de totale eiwitsynthese. Toevoegen van niet-gelabeld proline tijdens de incubatie leidt tot een sterke verhoging van de synthese van zowel niet-collagene als collagene eiwitten, hetgeen suggereert dat het prolinetransport een snelheidsbeperkende stap is. De collageensynthese in colon en ileum neemt significant af met de leeftijd van de rat. Een dergelijke afname is te verwachten op grond van bestaande literatuur over eiwit synthese bij veroudering en suggereert dat veranderingen welke in vivo in de darmwand plaatsvinden met deze in vitro bepaling kunnen worden gemeten.

De beschreven methode is vervolgens gebruikt om de collageensynthese te meten in darmnaden van de rat (**hoofdstuk 3**). Om dit proces te kunnen vergelijken in dunne en dikke darm worden zowel een ileumnaad als een colonnaad aangelegd in één rat. De dieren worden vervolgens na 3 of 12 uur, of na 1, 2, 3, 4, 7 of 28 dagen opgeofferd. In het ileum is de collageensynthese reeds 3 uur na operatie significant verhoogd. Deze blijft sterk verhoogd gedurende de eerste postoperatieve week met een maximale tienvoudige stimulering na 4 dagen. In het colon is de toename in synthesecapaciteit vertraagd vergeleken met die in het ileum; de maximale stimulering is hier zesvoudig. In bijna alle (48) ratten is de postoperatieve stimulering van de collageensynthese in het ileum hoger dan in het colon. Deze resultaten tonen aan dat het ileum sneller en sterker op verwondingen reageert dan het colon, althans wat de produktie van nieuw collageen betreft.

In het voorgaande experiment werden in elke rat twee darmnaden aangelegd om een directe vergelijking tussen ileum en colon mogelijk te maken. Vervolgens is nagegaan in hoeverre de constructie van één darmnaad, in ileum óf colon, per rat de collageensynthese in de rest van de darmwand beïnvloedt (**hoofdstuk 4**). Een of vier dagen na operatie is de synthese gemeten in de naad en op 10 andere lokaties, zes in de dunne darm en vier in de dikke darm. De resultaten worden vergeleken met waarden verkregen uit niet-geopereerde ratten. De collageensynthese in deze laatste groep blijkt door de dikke darm heen relatief uniform; in de intacte dunne darm is de synthese in de meest proximale en distale segmenten significant hoger dan in het tussenliggende weefsel. De postoperatieve verhoging van de collageensynthese blijkt niet strikt beperkt tot het (5 mm) naadsegment, maar is ook op afstand meetbaar. Dit

effect is, met name in de dunne darm, tot op zekere hoogte specifiek voor collageen omdat de synthese van niet-collageen eiwit aanzienlijk minder stijgt. Constructie van een naad in het colon heeft slechts een zeer beperkt effect op de collageensynthese in het ileum en vice versa. Dit betekent dat, indien twee naden binnen één rat worden aangelegd (zoals in de experimenten beschreven in **hoofdstuk 3**), het effect van de constructie van de eerste op de collageensynthese in de tweede te verwaarlozen is.

Een groep patiënten waarvan algemeen wordt aangenomen dat zij een verhoogde kans op naadlekkage vertonen is die welke rond de periode van operatie cytostatica krijgen toegediend. Eerder is op ons laboratorium gevonden dat in ratten die gedurende vijf achtereenvolgende dagen een combinatie van bleomycine, 5-fluorouracil en cisplatinum krijgen toegediend, en op de derde dag van deze kuur worden geopereerd, in de eerste postoperatieve week een sterke verlaging van de wondsterkte optreedt. **Hoofdstuk 5** bevat de resultaten van metingen van de collageensynthese in drie of zeven dagen oude darmnaden in ratten welke deze cytostatica intraveneus of intraperitoneaal krijgen toegediend. Het blijkt dat deze behandeling drie dagen na operatie resulteert in een ernstige, en specifieke, remming van de collageensynthese in zowel ileum- als colonnaden. De effecten van intraperitoneale toediening zijn significant groter dan die van intraveneuze toediening. Na zeven dagen is de collageensynthese in de intraveneus behandelde dieren terug op het niveau van de controlegroep terwijl synthese in de intraperitoneaal behandelde ratten nog steeds is geremd. Wij concluderen dat de verminderde sterkte van darmnaden het gevolg is van een cytostatica-geïnduceerde remming van de collageensynthese.

In de darmwand zijn verscheidene celtypen aanwezig die in staat zijn om collageen te produceren: fibroblasten, gladde spiercellen, endotheelcellen en zelfs macrofagen. Tijdens de wondgenezing zullen het waarschijnlijk met name de, naar het wondgebied migrerende, fibroblasten zijn die verantwoordelijk zijn voor de collageensynthese. In de volgende hoofdstukken worden een aantal experimenten besproken die de regulering van de collageensynthese in humane fibroblasten betreffen. Omdat er aanwijzingen zijn dat darmwonden in bepaalde situaties anders reageren dan huidwonden (bijvoorbeeld na het systemisch toedienen van sommige geneesmiddelen), worden hierbij steeds fibroblasten uit colon en huid vergeleken.

De negatieve effecten van cytostatica, beschreven in het vorige hoofdstuk, kunnen het gevolg zijn van verminderde migratie en proliferatie van fibroblasten. Het blijkt echter dat de onderscheiden verbindingen eveneens direct, en specifiek, de collageensynthese in fibroblasten kunnen remmen (**hoofdstuk 6**). De synthese in colonfibroblasten wordt sterk geremd door cisplatinum (in concentraties vanaf 10 μM), terwijl vergelijkbare molaire concentraties (tussen 0.1 en 50 μM) bleomycine en 5-fluorouracil nauwelijks effect vertonen. De collageensynthese in fibroblasten uit de humane huid wordt, dosis-afhankelijk, geremd door bleomycine maar niet door cisplatinum of 5-fluorouracil. Indien de collageensynthese wordt gestimuleerd met transforming growth factor-ß (TGFß), blijkt dat de additionele synthesecapaciteit door lagere concentraties cytostatica geremd wordt dan de basale synthesecapaciteit. Dit is een relevant gegeven omdat TGFß tijdens de wondgenezing in verhoogde mate tot expressie komt. In dit geval wordt de collageensynthese in huidfibroblasten, maar niet in colon fibroblasten, ook geremd door 5-fluorouracil. Deze resultaten geven aan dat een directe remming van de collageensynthese in de fibroblast de oorzaak kan zijn van vertraagde genezing van darmnaden die aangelegd worden tijdens een cytostaticakuur. Bovendien is het duidelijk dat fibroblasten uit huid en colon zeer verschillend kunnen reageren.

Dit laatste gegeven is verder uitgewerkt in **hoofdstuk 7**, waar de effecten van een aantal regulatoren op de collageensynthese in fibroblasten uit colon en huid worden vergeleken. Hierbij is gekeken naar serum, interleukine-1ß (IL-1ß), tumor necrosis factor (TNF), interferon-gamma, TGFß, dexamethason en de calcium-ionofoor A23187. Alle experimenten zijn verricht met cellen gekweekt in zowel aan als afwezigheid van foetaal kalfsserum. De duidelijkste verschillen tussen colon- en huidfibroblasten zijn dat

- de relatieve collageensynthese in huidfibroblasten toeneemt en in colonfibroblasten afneemt bij toevoegen van serum;
- IL-1ß in aanwezigheid van serum de collageensynthese remt in huidfibroblasten maar niet in colonfibroblasten;
- dexamethason de relatieve collageensynthese in huidfibroblasten remt maar niet in colonfibroblasten;
- TGFß in aanwezigheid van serum de collageensynthese in huidfibroblasten stimuleert en in colonfibroblasten remt.

Deze resultaten kunnen (een deel van) de verklaring vormen voor het gegeven dat het genezingsproces van wonden in huid en darm in verschillende mate wordt beïnvloed door bepaalde condities.

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CURRICULUM VITAE

Michael F.W.C. Martens werd op 24 juni 1961 geboren te Helmond. In 1981 behaalde hij het VWO diploma aan het Dr. Knippenberg college te Helmond. In datzelfde jaar startte hij met de studie Biologie aan de Universiteit van Amsterdam die succesvol werd afgesloten in juni 1987. Stages werden hierbij gelopen op de afdelingen Celbiologie van het Nederlands Kankerinstituut en Autoimmuunziekten van het Centraal Laboratorium van de Bloedtransfusiedienst, beide te Amsterdam. Tevens werd in deze periode de 1e graad onderwijsbevoegdheid behaald. In augustus 1987 werd een begin gemaakt, onder begeleiding van Dr. Th. Hendriks, met het in dit proefschrift beschreven promotie onderzoek, op het Researchlaboratorium van de afdeling Algemene Chirurgie van het St. Radboud Ziekenhuis te Nijmegen (Hoofd: Prof.Dr. R.J.A. Goris). Met ingang van 1 december 1991 is hij als wetenschappelijk medewerker werkzaam op de afdeling Research and Development van Nichols Institute Diagnostics B.V., te Wijchen.