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. Postoperative collagen synthesis, measured *in vitro* in intestinal anastomoses was studied from three hours to 28 days after operation. For this purpose, an ileal and a colonic anastomosis were constructed within the same animal and the results in both intestinal segments were compared. In the ileum, collagen synthesis was significantly increased, with respect to unoperated controls, three hours after operation. It remained raised during the period of study, with a maximal 10-fold stimulation four days after operation, and had nearly returned to the preoperative level after four weeks. The general pattern was the same in the colon, although quantitatively different: the increase in synthetic activity was delayed in comparison with the ileum. Maximal stimulation was approximately six-fold. In addition, we calculated the ratio for each rat between anastomotic collagen synthesis and the average value found in non-operated control animals. Postoperative stimulation in the ileum was higher than in the colon in almost every animal examined. The results show 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.

Dehiscence of intestinal anastomoses remains a major complication after surgery. Despite improved surgical techniques leakage of colonic anastomoses frequently occurs, resulting in high morbidity and mortality. In a much cited study on surgery 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 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 shown *in vivo*, in the ileum⁵ and in the colon⁶ 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 as the precursor needs to be injected at least 24 hours before death.⁶ A system to measure collagen synthesis in intestinal explants has recently been established.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 an ileal and a colonic anastomosis in the same animal and compared the postoperative course of collagen synthesis in both intestinal segments.

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 free access to water. The animals were killed by an intraperitoneal overdose of sodium pentobarbital and the intestinal biopsies were collected immediately.

MATERIALS

L-[2,3-³H]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 VII), deoxyribonucleic acid (calf thymus) and bovine serum albumin (BSA) were all obtained from Sigma, St-Louis,

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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, Darmstadt, Germany). Suture material used was ethilon 8x0 (Ethicon, Norderstedt, FRG).

OPERATIVE TECHNIQUES

All animals received an anastomosis in the ileum and the colon. Surgery was undertaken in semisterile conditions using a Zeiss operating microscope. The rats were anaesthetised by an intraperitoneal injection with sodium pentobarbital. The abdomen was opened through a midline incision of approximately 4 cm. The ileum was transected 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 transected 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 three and 12 hours and 1, 2, 3, 4, 7, 14, and 28 days the animals were killed (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 transection line) was isolated and cut into pieces of approximately 1–2 mm². 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 μ g/ml ascorbate and 250 μ g/ml kanamycin). Subsequently, 1.5 ml incubation medium was added and the samples were incubated for 30 min at 37°C (95% air; 5% CO₂). The medium was then removed by suction and replaced with 1.5 ml incubation medium containing 4.5 μ Ci [2,3-³H]proline. Incubation proceeded for three hours. All subsequent steps were carried out at 4°C. Both tissue and medium were transferred to a centrifugation tube and spun for five minutes at 2500 g. The sediment was homogenised in 3.0 ml 50 mM Tris-HCl, pH 7.6, containing 25 mM ethylenediaminetetraacetic acid (EDTA), 10 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulphonylfluoride (PMSF) and 1 mM proline. Trichloroacetic acid (TCA) was added (final concentration 0.6 M) to the homogenate which was then centrifuged for five minutes at 2500 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 neutralised by the addition of 0.3 ml 1 M N-(2-hydroxyethyl)piperazine-N-(2-ethanesulphonic 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 CaCl₂ and 0.1 ml collagenase (chromatographically purified on a G200 gel filtration column) were added to a 0.5 ml aliquot of the solubilised sample and the mixture was incubated for five hours at 37°C. The digestion was terminated by the addition of trichloroacetic acid and tannic acid up to final concentrations of 0.6 M and 3 mM, respectively. After centrifugation (10 min; 14 500 g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyser. 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 collagenase digestible protein fraction from that in total protein yields the incorporation into non-collagenous protein (NCP).

The relative collagen synthesis was calculated with the formula⁹ that takes into account the enrichment of proline in collagen compared with other proteins:

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

OTHER PROCEDURES

DNA was measured in the NaOH-solubilised trichloroacetic acid sediment, by the method of Burton¹⁰ using calf thymus DNA as a standard. Statistical methods are mentioned with the results.

Results

The intestinal wall synthesises 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 four day old anastomoses, constructed both in the ileum and the colon, were incubated with [³H]proline for various time periods. Incorporation into the collagenase digestible protein fraction increased linearly with time and tissue weight (results not shown). The standard conditions chosen for subsequent experiments, 50 mg tissue incubated for three hours, fall well within the linear part of the respective curves.

Collagen synthesis around intestinal anastomoses is strongly enhanced as compared with 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

TABLE I Collagen synthesis in ileal anastomoses

Time after operation	Collagenase digestible protein dpm/mg wet weight	Collagenase digestible protein dpm/ μ g DNA	Relative collagen synthesis (%)
3 h	177 (69) [†]	57 (20)*	0.36 (0.09)
12 h	262 (68) [†]	71 (19) [†]	0.43 (0.08)*
1 d	277 (87) [†]	108 (52)*	0.52 (0.10) [†]
2 d	310 (88) [†]	101 (41)*	0.61 (0.17) [†]
3 d	509 (82) [†]	167 (64) [†]	1.09 (0.21) [†]
4 d	687 (268) [†]	300 (126) [†]	2.42 (0.72) [†]
7 d	284 (104) [†]	184 (120) [†]	1.69 (0.67) [†]
28 d	109 (32)	70 (32)	0.54 (0.25)
Control	70 (18)	41 (7)	0.30 (0.04)

Explants from anastomotic tissue were incubated for three hours with 4.5 μ Ci [3 H]proline. Results are expressed as dpm collagenase digestible protein 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's test: *0.01 < p $\leq$ 0.05; [†]p $\leq$ 0.01.

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, in preparation), precluded the use of control segments removed at time of death. Because 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 the ileum or the colon and collagen synthesis was measured after four 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/ μ g DNA, compared with 41 (17) dpm CDP/ μ g DNA in ileum of unoperated control animals. Likewise, collagen synthesis was 52 (11) dpm CDP/ μ g DNA in intact colon in rats with an ileum anastomosis and 77 (8) dpm CDP/ μ g DNA in colon of unoperated controls.

TABLE II Collagen synthesis in colonic anastomoses

Time after operation	Collagenase digestible protein dpm/mg wet weight	Collagenase digestible protein dpm/ μ g DNA	Relative collagen synthesis (%)
3 h	267 (77)	73 (20)	0.62 (0.14)
12 h	263 (35) [†]	88 (16)	0.64 (0.10)
1 d	370 (21) [†]	123 (40)	0.88 (0.14) [†]
2 d	399 (67) [†]	126 (48)*	0.93 (0.11) [†]
3 d	1002 (462) [†]	253 (88) [†]	2.65 (1.23) [†]
4 d	1086 (375) [†]	373 (188) [†]	2.87 (0.72) [†]
7 d	421 (148) [†]	154 (55) [†]	1.48 (0.51) [†]
28 d	192 (85)	108 (36)	1.01 (0.24) [†]
Control	188 (29)	77 (8)	0.52 (0.03)

Explants from anastomotic tissue were incubated for three hours with 4.5 μ Ci [3 H]proline. Results are expressed as dpm collagenase digestible protein 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's test: *0.01 < p $\leq$ 0.05; [†]p $\leq$ 0.01.

Figure 1: Relative increase of collagen synthesis in anastomotic explants from both ileum and colon. In each anastomosis collagen synthesis was measured as dpm collagenase digestible protein/mg wet weight (A), dpm CDP/ μ g 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 (SD) from six experimental animals.

Collagen synthesis was measured from three hours to 28 days after operation. In Table I the average values for ileal anastomoses are given. Three hours after operation incorporation of label into collagenase digestible protein, expressed on a 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 three hours to four days after operation the absolute and the relative collagen synthesis rose steadily. Thereafter, synthetic activity started to fall: the average values in two five day old anastomoses were 230 dpm collagenase digestible protein/mg wet weight, 132 dpm/ μ g DNA, and 1.73% relative collagen synthesis, respectively. After four weeks collagen synthesis in the anastomotic area had nearly returned to the preoperative level; average values not being significantly different from those measured in the controls.

The general pattern was the same in the colon (Table II), although the increase in synthetic activity appeared delayed in comparison with the ileum. The absolute collagen synthesis was significantly raised after 12 hours, if expressed on a wet weight basis, and only after two 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 the colon, synthesis also seemed maximally stimulated after four days. The average values measured in two five day old colonic anastomoses were 288 dpm

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/ μ g 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).

collagenase digestible protein/mg wet weight, 151 dpm collagenase digestible protein/ μ g DNA and a relative collagen synthesis of 1.39%, respectively. Values after four weeks were not significantly different from control values, except for the relative collagen synthesis which was still raised.

We then 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 the ileum than in the 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, Fig 1A). In contrast, the stimulation of the relative collagen synthesis appeared comparable in both small and large bowel, certainly up to two days (Fig 1C). Three days after operation, the average ratio in the colon was even higher than in the ileum, although at four and seven days the effect was again the other way around.

In order to further substantiate the idea that

anastomotic construction stimulates collagen synthesis more strongly in the ileum than in the colon, the relative increase in both segments was compared within each rat. For 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 the ileum than in the colon. The data for all rats are represented in Figure 2. Because the number of rats killed at each time point is relatively small (six), the ileum-colon difference is only significant (one-sided signed rank test) if a ratio above 1 is found in all six animals. For the synthetic activities, expressed on a wet weight basis (Fig 2A) this was the case at 12 hours and two days after operation. Still, at the other time points a ratio above 1 was observed in five of six animals. Altogether, the reverse – a ratio lower than 1, indicating a stronger stimulation in colonic anastomoses – was the case in only six of 48 animals. This effect was even more pronounced if synthetic activity was expressed on the basis of DNA (Fig 2B). Here, a significant effect was noted at 12 hours, one day, four days, and seven days after operation. A different picture emerged for the relative collagen synthesis (Fig 2C): no consistent difference was noted between the ileum and the colon. Stimulation of collagen synthesis in ileal anastomoses was consistently and significantly higher than in colonic anastomoses at seven days after operation only.

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 post-operative 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. Because 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 used 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 assess 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, nine-fold in the ileum and six-fold in the colon as compared with uninjured intestine, is similar in both types of experiments.

So far, no data have been published concerning anastomotic collagen synthesis during the first two days of the healing period. Clearly, synthesis is enhanced very quickly after wounding, certainly in the ileum where incorporation of [³H]proline into CDP has risen significantly already after three hours. As the influx of fibroblasts, which are generally believed to be the source of collagen produced during wound healing, is only apparent two days after anastomotic construction¹⁴ 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 synthesising type I, III, and V collagen.^{15,16} The authors suggest that these cells could play an important role in repair processes after damage and inflammation of the intestinal wall.

Apart from 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 [³H]hydroxyproline/ μ mol hydroxyproline) and measured four 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/ μ g DNA, in ileal and colonic anastomoses at this time point (Tables I and II). Such an approach, however, does not take into consideration the fact⁷ that basal synthesis in colon is significantly higher than in the 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 collagenase digestible protein, expressed on the basis of wet weight or DNA, postoperative stimulation in the ileum is higher than in the colon in almost every animal examined (Fig 2). Moreover, ileal synthesis is clearly stimulated at an earlier stage than synthesis in colon. For

instance, incorporation of [³H]proline into collagenase digestible protein and expressed per μ g DNA, is significantly higher than in unoperated controls at three hours after operation in the ileum and at 48 hours after operation in colon. Taken together, we believe that these results indicate 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^{4,8} which showed that the transient post-operative 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 – for example, platelet derived growth factors – in the immediate post-operative period. Because the preoperative level of collagen synthesis is higher in the colon, it could be that the cells in question reside at a higher level of activity than those in the ileum and therefore need a greater stimulus in order to increase their collagen production.

It is generally accepted that anastomotic collagen determines anastomotic strength, and next to synthesis, degradation of this matrix protein will affect wound strength. Production of collagenase has been shown immunohistochemically around colonic anastomoses¹⁹ 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 emphasised 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 postoperative collagen synthesis, observed between ileal and colonic anastomoses constructed under normal conditions, 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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