

# Rapid morphological changes and loss of collagen following experimental acute colonic obstruction

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## Abstract

**Purpose** Anastomosis of an acutely obstructed colon is associated with an increased risk of dehiscence. In experimental models, acute obstruction decreases collagen in the colonic wall, but the time course and propagation along the colon of the biochemical changes are unknown. Furthermore, there is a paucity of information on the correlation between these biochemical changes and histological features.

**Methods** Forty male Sprague Dawley rats were subjected to partial obstruction by placing a silicone ring around the left colon 30 mm above the reflection. Obstruction was maintained for 0, 1, 2, 3 or 4 days. Samples from five different locations along the colon were analysed on circumference, tissue water content, collagen concentration and histomorphology. Neutrophil and macrophage infiltration was characterized immunohistochemically.

**Results** The colonic circumference and water content increased ( $p < 0.001$ ), while the collagen concentration decreased by 48 %

( $p < 0.01$ ) proximal to the obstruction already after 1 day. The degree of dilation and collagen reduction did not change significantly over the subsequent 3 days of obstruction, whereas the water content normalized by day 3. Mucosal and submucosal oedema and the relative neutrophil infiltration were highest after 1 day in the colonic segment proximal to the stenosis while the macrophage population continued to increase to day 4. Muscular necrosis in addition to ganglionitis and neuritis in the nervous plexus increased with duration of obstruction.

**Conclusions** The pronounced and rapid changes of the composition of cells and the extracellular matrix of the colonic wall following acute obstruction may be of guidance for present surgical treatments and future pharmacological interventions.

**Keywords** Colonic obstruction · Collagen · Histology · Inflammation · Neutrophil · Macrophage

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## Introduction

Colorectal cancer is one of the most frequent malignancies worldwide with over 1.2 million new cases annually [1]. Approximately 15 % present as acute obstructing tumours interrupting the bowel continuity [2] and require prompt treatment. These emergency operations are high-risk procedures [3, 4]. Despite progress in surgical techniques, moving from the staged procedures to resection with construction of a primary anastomosis [4, 5], the presence of colonic obstruction is associated with an increased risk of anastomotic leakage [6, 7] and mortality [8]. Unfortunately, Hartmann's procedure [9, 10] and endoscopic placement of a self-expandable metal stent [11–13] are both associated with drawbacks as well. Regardless of surgical technique, a detailed knowledge of the biochemical and structural changes in the obstructed colonic wall is crucial to better understand the pathophysiology behind and the association with increased anastomotic leakage.

In a non-obstructed colon, anastomotic healing comprises a cascade of events, aiming to restore the function and biomechanical strength in the bowel. The submucosal collagen constitutes the suture-holding tissue and ensures anastomotic integrity in the first postoperative week [14]. During this period, collagen degradation predominates over collagen synthesis resulting in decreased concentration of collagen and reduced anastomotic biomechanical strength [15]. In the obstructed colonic wall, reduced collagen has been observed in rodents [16–19], but the time course as well as propagation of changes along the colon following obstruction has not been addressed previously.

The purpose of the present study was to examine the dimensional, biochemical and morphological changes in the colonic wall following 0 to 4 days of obstruction using an established acute model in rats [17–19].

## Methods

Forty male Sprague Dawley rats (Taconic M&B, Ry, Denmark) weighing  $348 \pm 61$  g (mean  $\pm$  SD) were acclimatized for a minimum of 7 days prior to surgery with free access to standard pellets and tap water [15, 17]. The rats were randomized into five groups of eight rats in each group and subjected to partial colonic obstruction for 0, 1, 2, 3 or 4 days. After surgery, four rats were kept per cage. The study was approved by the local ethics committee for animal studies at Lund University (M174-04).

### Anaesthesia and analgesics

Anaesthesia was induced with a mixture of 0.14 mg/kg fentanyl citrate and 4.4 mg/kg fluanisone (Hypnorm®; Janssen-Cilag, Beerse, Belgium), and 2.2 mg/kg midazolam (Dormicum®; F. Hoffmann-La Roche, Basel, Switzerland) given subcutaneously. After the surgery was completed, 0.02 mg/kg buprenorphine (Temgesic®; Schering-Plough, Brussels, Belgium) was injected subcutaneously. This regimen was also used for postoperative analgesia.

### Surgery

The abdominal cavity was exposed through a 30-mm midline incision. Partial colonic obstruction was established using a 5-mm-wide silicone ring (6.5 mm inner diameter) placed around the left colon between two marginal veins 30 mm above the peritoneal reflection [17–19]. The ring was closed with single 7-0 polypropylene suture (Ethilon®; Ethicon, Johnson & Johnson, Brussels, Belgium). The abdomen was closed in two separate layers using continuous 4.0 sutures (Vicryl®; Ethicon).

The rats were killed by asphyxiation in a carbon dioxide chamber after 0, 1, 2, 3 or 4 days of colonic obstruction. Five-millimetre-wide segments of the colon were excised at five

predefined anatomical locations. Segment A was located 10 mm distal to the ileocolonic junction; segment B was 15 mm and segment C 5 mm proximal to the stenotic silicone ring, both segments 5 mm apart, and segment D 5 mm and segment E 15 mm distal to the ring as detailed in Fig. 1. The segments were cut open at the antimesenteric border, and the colonic circumference was measured in millimetres using a slide caliper. Each segment was bisected for determination of water and collagen contents (segments A, B, C, D and E) and for histopathological (segments C, D and E) and immunohistochemical examination (segments C and D).

### Water content and collagen (hydroxyproline) analyses

Tissues were weighed ( $m_{\text{Fresh}}$ ), dried to constant weight at 100 °C and weighed ( $m_{\text{Dry}}$ ). Tissue water content was calculated as:  $(1 - (m_{\text{Dry}}/m_{\text{Fresh}})) \times 100 \%$ .

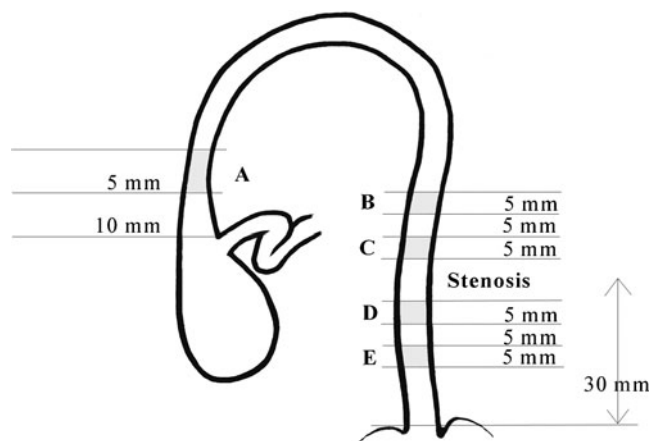
Tissues were hydrolyzed in 6 M hydrochloric acid for 18 h at 110 °C. The acid hydrolysates were evaporated and the washed, acid-free residues dissolved in acetate–citrate buffer [19]. The hydroxyproline content was determined colourimetrically [19].

### Histological evaluation

Colonic specimens were fixed in 4 % phosphate-buffered paraformaldehyde for 24 h and embedded in paraffin. Sections (5  $\mu\text{m}$ ) were stained with haematoxylin–eosin. A consultant specialised in gastrointestinal pathology evaluated the sections without prior knowledge of group affiliation. Oedema was graded as no, slight or pronounced. The degree of inflammatory cell infiltration was evaluated on a four-graded scale [20].

### Immunohistochemical double labelling of neutrophils and macrophages

Tissue sections (5  $\mu\text{m}$ ) were deparaffinized, rehydrated and microwaved for 10 min in Tris–ethylenediaminetetraacetic



**Fig. 1** Colonic sampling sites

acid (pH 9.0). Double labelling of neutrophils and macrophages was performed using reagents and protocols from Vector Laboratories (Burlingame, CA, USA) unless stated otherwise. Each incubation step was carried out at ambient temperature and was followed by washing in phosphate-buffered saline (pH 7.2) for 5 min.

Endogenous peroxidase activity was quenched with 3 % hydrogen peroxide for 5 min. Sections were then incubated with 2.5 % normal horse serum for 20 min. The rabbit anti-rat neutrophil adsorbed antiserum (AIAD51140; Accurate Chemical & Scientific Corporation, Westbury, NY, USA) [21] was diluted 1:10,000 in 1 % bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and applied for 60 min. The section was then incubated with the anti-mouse/rabbit Ig ImmPRESS peroxidase universal reagent for 30 min. The bound antibody complex was visualized with 3,3'-diaminobenzidine supplemented with nickel chloride for 4 min resulting in grey/black-labelled neutrophils. The tissue section was blocked again with 2.5 % normal horse serum for 20 min. The mouse monoclonal anti-rat CD68 antibody (clone ED1; Serotec, Oxford, UK) [22] was applied at 1:400 dilution in 1 % bovine serum albumin for 60 min. Following 30-min incubation with the anti-mouse/rabbit Ig ImmPRESS peroxidase universal reagent, ImmPACT NovaRED was applied to the section for 2 min resulting in red-labelled macrophages. Sections were counterstained with Mayer's haematoxylin (blue) for 40 s, cleared and mounted using Pertex mounting medium (Histolab Products, Göteborg, Sweden).

## Statistics

Hydroxyproline levels were log-transformed to obtain normal distribution. One-way ANOVA with Newman-Keuls was applied for comparisons with day 0. A  $p < 0.05$  was considered statistically significant. Hydroxyproline data are given as geometric mean  $\pm$  back-transformed standard error (SE) and the other variables as mean  $\pm$  SD.

## Results

We have characterized the temporal and histological changes in the colonic wall following acute mechanical obstruction using a model developed in our laboratory [17–19]. There were no differences in preoperative body weight ( $p=0.45$ ) among the five groups. One day-3 rat died during recovery from anaesthesia leaving 39 rats for the analyses. The partially obstructed colon allowed passage of flatus but not faeces, and animals continuously lost weight throughout the study period from  $8 \pm 9$  g after day 1 to  $33 \pm 6$  g after 4 days of obstruction.

## Colonic circumference

After 1 day of obstruction, the colonic circumference increased in segment B from  $8 \pm 1$  to  $17 \pm 2$  mm ( $p < 0.001$ ) and in segment C from  $8 \pm 2$  to  $19 \pm 2$  mm ( $p < 0.001$ ) (Fig. 2). The colonic dilation did not increase further with duration of obstruction. The colonic circumference of segment A increased from  $6 \pm 1$  mm before obstruction to  $12 \pm 3$  mm ( $p < 0.01$ ) after 4 days of obstruction. The circumference of distal segment D did not change significantly over time whereas the circumference of segment E decreased after 2 days of obstruction from  $11 \pm 3$  mm on day 0 to  $8 \pm 1$  mm on day 4 ( $p < 0.01$ ).

## Colonic water content and collagen

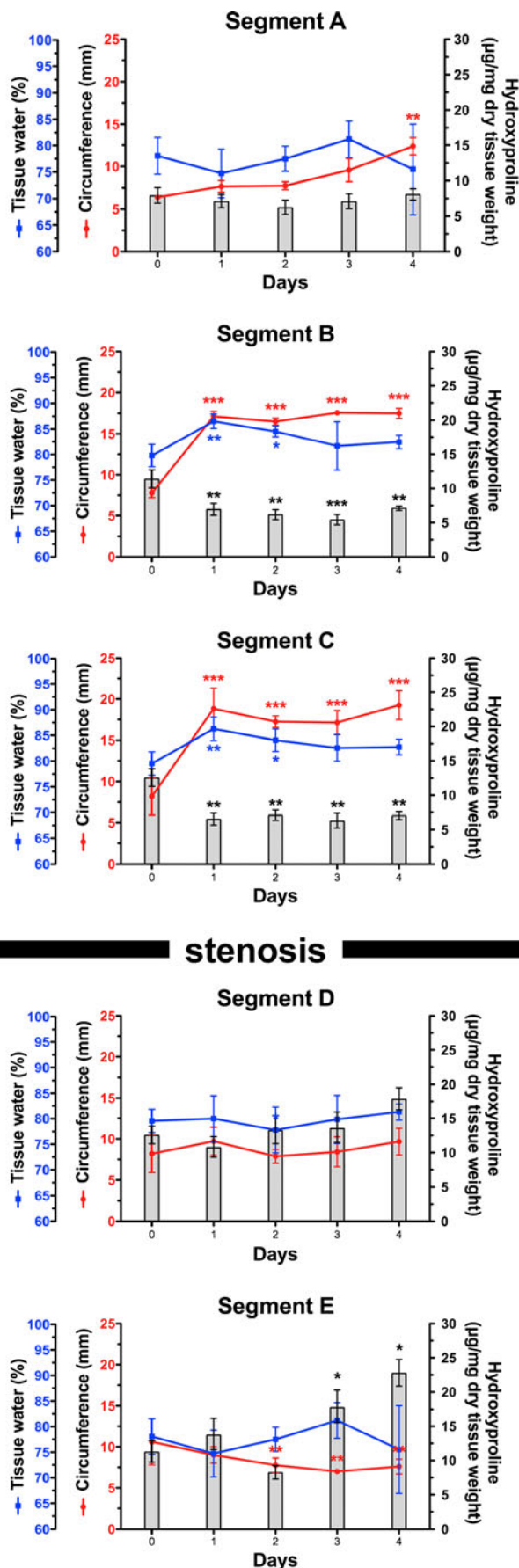
The percentage of water in the colonic wall of segment B increased from  $80 \pm 2$  % on day 0 to  $86 \pm 3$  % on day 1 ( $p < 0.001$ ) and to  $83 \pm 1$  % on day 2 ( $p < 0.05$ ) but returned to day-0 levels days 3 and 4 (Fig. 2). A similar pattern was observed in segment C, whereas the water content in segments A, D and E did not change with time.

The collagen concentration decreased in segment B after 1 day of obstruction by 39 % from  $11.3 \pm 1.4$  to  $6.9 \pm 0.9$   $\mu\text{g}$  hydroxyproline/mg dry weight ( $p < 0.01$ ) (Fig. 2). The corresponding value for segment C was 48 % from  $12.5 \pm 1.3$  to  $6.5 \pm 0.9$   $\mu\text{g}$ /mg dry weight ( $p < 0.01$ ). The collagen concentration did not significantly change further after day 1 in either segment B or C. In segment E, collagen levels increased from days 0 to 3 by 58 % from  $11.2 \pm 1.7$  to  $17.7 \pm 2.5$   $\mu\text{g}$ /mg dry weight ( $p < 0.05$ ) and from days 0 to 4 by 102 % to  $22.7 \pm 2.0$   $\mu\text{g}$ /mg dry weight ( $p < 0.01$ ). The collagen levels did not change significantly with duration of obstruction in segment A or D.

## Histological and immunohistochemical examinations

These analyses are presented in relation to day 0 (Fig. 3a). Immunohistochemical staining was applied to specifically identify neutrophils and CD68-positive macrophages in selected sections (Fig. 4a and b).

*Day 1* The mucosa of the proximal segment C was slightly oedematous and infiltrated with neutrophils. The CD68 immunoreactivity in the mucosa did not change appreciably with obstruction. The muscularis mucosa was thickened with scattered necroses. The submucosa exhibited a pronounced oedema and a moderate infiltrate composed mostly of neutrophils with fewer macrophages, lymphocytes and plasma cells (Figs. 3b and 4c). The submucosa of distal segment D was slightly oedematous but less populated by neutrophils than the C segment (Fig. 4e and g).



**Fig. 2** Colonic inner circumference (red curves, mean  $\pm$  SD), tissue water in percent (blue curves, mean  $\pm$  SD) and hydroxyproline concentration (bars, geometric mean  $\pm$  back-transformed SE) in the five segments of the colonic wall (see Fig. 1) after 0 to 4 days of obstruction. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 compared to day 0

*Day 2* The mucosal oedema had diminished, while the inflammation persisted. In the submucosa, the oedema was unaltered from day 1, and the inflammatory cell infiltration was moderate. At this stage of obstruction, scattered fibrinoid necroses and thrombosed arteries were apparent. The longitudinal layer of the tunica muscularis was moderately inflamed with scattered necroses. In the myenteric plexus of Auerbach, lymphocytes were found in the ganglions. Ganglionitis was evident in the proximal segment C and, to a lesser degree, in the distal segment D.

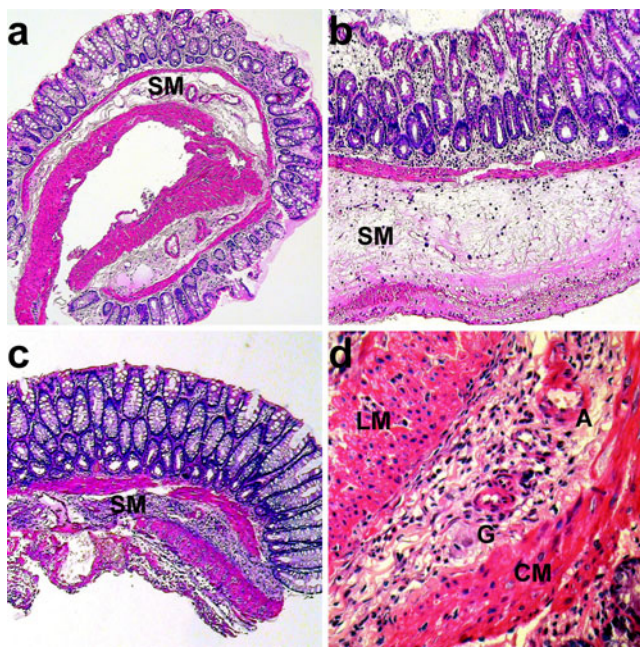
*Days 3 and 4* Occasional neutrophils were observed in the mucosa of the proximal segment. The submucosal oedema decreased leading to apparently increased cell densities. The inflammatory cell infiltrate peaked on day 3 (Fig. 3c). Although the proportional number of neutrophils declined progressively proximally to the stenosis, it still exceeded that of the distal D segment on day 4 (Fig. 4g). In contrast, the proportion of CD68-positive macrophages was increased and encompassed about half of the cell count in the submucosa both above and below the obstruction on day 4 (Fig. 4d, f and g). Pronounced neuritis with neutrophils and lymphocytes in close vicinity to the neurons in the circular layer of the tunica muscularis was noted on either side of the stenosis. Ganglionitis persisted, and the neurons began to degenerate although necrotic neurons were only demonstrated in the proximal segment C (Fig. 3d). Severe peritonitis was present in the serosa of segment C. There were no obvious histological changes with time in segment E.

## Discussion

The collagen-rich submucosa of the colonic wall constitutes the suture-bearing capacity and is paramount for the biomechanical strength of an anastomosis [14]. The present experimental study demonstrates that acute colonic obstruction dramatically and rapidly decreases collagen levels within the first day of obstruction. Interestingly, the kinetics of this collagen loss is analogous to that of the rat uterus during postpartum involution [23]. The early changes in the colon correlated with bowel dilation and pronounced oedema proximal to the obstruction.

Furthermore, neutrophils accumulated proximal as opposed to distal to the stenosis in the submucosa. We did not anticipate this rapid cellular response, and it is possible that the influx of neutrophils peaked even before 1 day of obstruction. On the other hand, the finding is consistent with the significantly higher



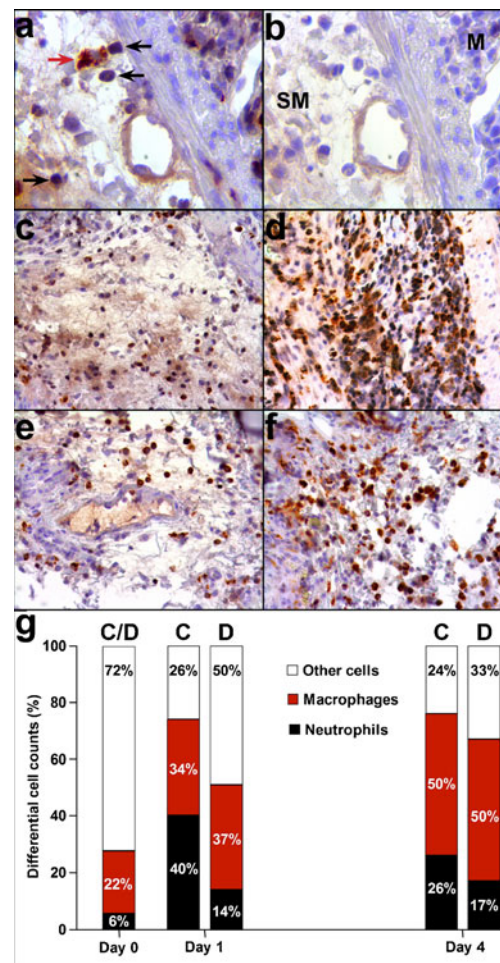


**Fig. 3** Morphologic changes of the proximal segment of the colon with time of obstruction from day 0 (a), 1 (b), 3 (c) and to day 4 (d). Note mucosal and submucosal oedema with inflammatory cell infiltrate on day 1 (b), while at day 3 (c), inflammation was severe in the submucosa with serosal peritonitis but with less submucosal oedema. **d** Pronounced ganglionitis was observed on day 4. *A* artery with adjoining extravasated neutrophils, *G* ganglionitis, *LM/CM* longitudinal/circular layers of the tunica muscularis, *SM* submucosa. Original magnifications: **a**  $\times 100$ ; **b** and **c**  $\times 200$ ; **d**  $\times 600$ . Haematoxylin–eosin stain

myeloperoxidase levels previously demonstrated in the colonic wall proximal to the stenosis on day 4 [17].

Collagen degradation is accomplished by the action of one or more matrix metalloproteinases (MMPs). We have previously shown that the reduced collagen in an obstructed colon correlates with MMP activity [17], which tended to increase with the degree of colonic dilation [19]. Neutrophils primarily express MMP-8 and MMP-9, which collectively degrade the colon and type I collagens synergistically [24]. Neutrophils were probably the major cellular sources of MMPs here. To test this hypothesis, the recruitment of neutrophils could be blocked experimentally by giving specific antibodies against the adhesion molecule CD18 [25] or perhaps against responsible chemoattractants such as lipopolysaccharide-induced CXC chemokine [26]. Intuitively, the high mechanical load would increase the susceptibility of collagen to degradation by MMPs, although the opposite has been reported in vitro [27].

We suggest that the colonic wall proximal to an obstructing lesion is depleted of collagen, which impairs the healing conditions of anastomoses [14, 28]. Broad-spectrum MMP inhibition restores anastomotic integrity in a non-obstructive colon [15, 20], but it remains to be demonstrated whether this therapeutic approach is beneficial for anastomotic repair in the obstructed colon as well.



**Fig. 4** Immunohistochemical double labelling of neutrophils (black) and macrophages (red) in rat colon proximal (a–d) and distal (e, f) to the obstructive ring applied for 1 day (a–c, e) or for 4 days (d, f). **a, b** High-resolution (oil) images of adjacent tissue sections treated with (a) or without (b) the primary antibodies directed against neutrophils (a black arrows) and macrophages (a red arrow). **b** Submucosa (*SM*) and mucosa (*M*) are indicated for the purpose of orientation. **c–f** Note the apparent sparse cellularity on day 1 due to extensive oedema but increased cell density in proximal versus distal segments. Original magnifications: **a** and **b**  $\times 1,000$ , **c–f**  $\times 400$ . Mayer's haematoxylin counterstain. **g** Differential counts of neutrophils, macrophages and other cells (endothelial cells excluded). Cells were counted manually in digital images ( $\times 400$ ) of representative areas of the entire thickness of the submucosa in comparison with day 0. *C* and *D* above bars refer to the proximal and distal segments to the obstruction

With time, the macrophage infiltration increased in the submucosa of the stenotic area. This may reflect an increased demand for macrophages in protective and reparative processes [29].

It is striking that, after the initial reduction, the collagen concentration proximal to the stenosis was maintained at the same low level. This finding suggests that degraded collagen was compensated for by the synthesis of new collagen molecules. In general, mechanical tension induces a synthetic fibroblast phenotype [30] under experimental conditions

that mimic the increased strain exerted on the fibroblasts and myofibroblasts in the dilated colon.

Colonic dilation progressed to the most proximal segment of the colon after 4 days of obstruction. In contrast, the circumference of the most distal segment examined decreased, resulting in increased collagen concentration. The mechanisms are elusive because, in experimental studies on diverting stoma, the collagen concentration in the offloaded colon did not change [31]. Moreover, the chronically obstructed small intestine exhibits increased collagen levels and muscle hypertrophy [32], none of which was demonstrated in the present study.

We did not investigate the ultrastructural arrangements of the collagen fibrils [33] or the specific types of collagen although the submucosa is composed primarily of type I and III collagens [34, 35]. Apart from collagen, hydroxyproline is also found in elastin, but the contribution of elastin-derived compared with collagen-derived hydroxyproline is negligible [36]. It would be interesting to study the changes of other extracellular components such as fibronectin and laminin during colonic obstruction [34].

The scattered necroses observed after 4 days of obstruction in the muscular layer of the colonic wall might inhibit the return of bowel function as we have previously observed [19]. Neural damage including neuritis and ganglionitis in the tunica muscularis, shown here, may impair bowel peristaltic movement and prolong postoperative ileus.

In conclusion, the early and dramatic loss of structural collagen in the large bowel may hint that caution should be taken regarding primary anastomosis, also in patients with only a short history of colonic obstruction. Moreover, our results indicate that it would be worthwhile to test if the loss of connective tissue elements is preventable by pharmacologic means to reduce the risk of anastomotic leakage.

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