Intraoperative Irradiation Delays Anastomotic Repair in Rat Colon

Wilfried F. Seifert, PhD, Theo Wobbes, MD, PhD, Jaap Hoogenhout, MD, PhD, Ben M. de Man, Karin M.L.C. Huyben, Thijs Hendriks, PhD, *Nijmegen, The Netherlands*

BACKGROUND: **There exists a growing interest in intraoperative radiation therapy as a treatment modality for large-bowel cancer. Since such therapy could interfere with wound repair, we investigated its effects on early healing of colonic anastomoses.**

METHODS: **After resection of 1 cm of colon, rats were irradiated with a single dose of 25 Gy, either to the proximal limb (P group) or to both proximal and distal limbs of the bowel (PD group) before anastomotic construction. Both groups were compared with a sham-irradiated control group. Animals were killed 3, 7, or 14 days after operation, and healing was assessed by mechanical and biochemical (collagen) parameters.**

RESULTS: **Three days after operation, bursting pressure was significantly lowered in the P group, whereas in the PD group both bursting pressure and breaking strength were strongly reduced. At day 7, the breaking strength was still reduced in the PD group, but not significantly so in the P group. The collagen synthetic capacity of the anastomotic segments was significantly lowered in both irradiated groups at day 3, resulting in a diminished collagen concentration in the actual wound area after 7 days. At 14 days after operation, no differences in strength were found between control and irradiated groups, while anastomotic hydroxyproline levels were significantly higher in both the P and PD groups than in the control group.**

CONCLUSIONS: **High-dose intraoperative radiation therapy delays the healing of colonic anastomoses; it transiently reduces strength, probably as a result of a diminished accumulation of collagen.** *Am J Surg.* **1995;170:256–261.**

From the Departments of Surgery (WFS, TW, BMdM, KMLCH, TH), and Radiotherapy (JH), University Hospital Nijmegen, Nijmegen, The Netherlands.

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Requests for reprints should be addressed to Wilfried F. Seifert, PhD, University Hospital Nijmegen, Department of Surgery, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

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Radiation therapy is frequently used as an adjuvant to surgery in cancer patients.[1] Many treatment protocols have been devised in which fractionated irradiation is delivered to the tumor bed over the weeks preceding or following surgery. Intraoperative radiation therapy has been a subject of recent interest as a valuable innovation in large-bowel cancer therapy.[2-4] Following surgical resection of the tumor, the residual tumor volume and tumor bed are directly given a single moderate-to-large dose of radiation. Compared with conventional fractionated external radiation therapy, the benefit of this procedure is the accurate localization of the site to be irradiated while the risk of radiation damage to surrounding normal tissues is minimized by shielding and surgical mobilization. The combination of colorectal surgery and intraoperative radiation therapy, however, is associated with definite risks of complications to the intestine, since the remaining distal rectum should not be shielded and has to be incorporated into an anastomosis. Therefore, it becomes imperative to investigate the effects of such irradiation on anastomotic repair because suppressed healing could increase the risk for anastomotic dehiscence, which is an extremely serious complication with a concomitant high mortality rate.[5,6] So far, no data are available on the effects of intraoperative radiation therapy on the repair of large-bowel anastomoses.

During the first postoperative days anastomotic strength is low, and therefore chances for dehiscence are relatively high. The postoperative collagen metabolism is crucial to anastomotic repair because the developing strength of the sutured intestinal wall is derived to a large extent from collagen fibrils in the submucosa.[7,8] Early anastomotic strength depends on the ability of the existing collagenous network to retain the sutures while newly formed collagen fibrils should restore the original strength to the healing bowel. The formation of cross-links between the newly formed collagen molecules ultimately decides the stability of the fibrils.[9] Thus, postoperative collagen content, synthesis, and cross-linking may strongly affect anastomotic strength.

In this study, we investigated the effects of intraoperative irradiation, delivered to one or both of the resection edges, on the development of anastomotic strength and the metabolism of collagen in large-bowel anastomoses in the rat.

MATERIALS AND METHODS

Operative Procedure

Male outbred Wistar/Cpb:WU rats, 3 months old and weighing 285 ± 15 g (mean ± standard deviation [SD], n = 216), were obtained from our own colony (Nijmegen, The Netherlands). The animals were housed in groups of 3 in Makrolon type 3 cages. Water and a standard laboratory

chow (Diet AM II, Hope Farms, Woerden, The Netherlands) were supplied ad libitum. The study was approved by the Animal Ethical Review Committee of the Faculty of Medicine, University of Nijmegen.

After a 7-day preexperimental period, the rats were randomly divided into 3 groups of 72 rats: a control group that underwent a sham-irradiation procedure before anastomotic construction, a group (P) where the proximal resection edge of the bowel was irradiated with a dose of 25 Gy before anastomotic construction, and a group (PD) where both the proximal and distal resection edges of the bowel were irradiated before construction (**Figure 1**). Body weight was recorded daily. All signs of illness, reaction to treatment, and mortality were recorded. Ten animals of each group were killed at 3, 7, and 14 days after surgery to determine anastomotic strength, hydroxyproline content, and hydroxyproline solubility. At the same days, similar groups of animals (n = 10 each) were killed to determine the collagen concentration of the wound area by morphometry in Sirius-red–stained sections (Sirius-Red F3BA, Chroma, Köngen, Germany). Six animals of each group were killed at days 3 and 7 to measure the anastomotic collagen synthetic capacity.

At the day of operation, the rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Surgery was performed under semisterile conditions using a Zeiss operation microscope. The abdominal skin was shaved, disinfected with 70% v/v ethanol, and opened by a median laparotomy of 4 cm. In each animal, 1 cm of colon was resected 3 cm proximal to the rectal-peritoneal reflection. Intraoperative irradiation was performed on the limb or limbs held apart in a lead cone to prevent unwanted irradiation of adjacent tissue as described before.[10] In the P group, 2 cm of the proximal resection end was irradiated; and in the PD group, 1 cm of both the distal and proximal resection ends was irradiated (Figure 1). A dose of 25 Gy was delivered by a 250 kV x-ray unit with a 1-mm Cu filter at a dose rate of 1.29 Gy per minute. The control group underwent a sham-irradiation procedure before anastomotic construction. An end-to-end anastomosis was constructed using 8 single-layer inverting interrupted 8 × 0 Ethilon sutures (Ethicon, Norderstedt, Germany). The abdomen was closed using a 3 × 0 silk suture for the fascia and staples for the skin.

Anastomotic Strength

After 3, 7, or 14 days, the animals were killed by an overdose of pentobarbital sodium. The abdomen was opened and inspected for signs of intestinal leakage. The anastomotic segment was resected and washed in saline, and bursting pressure and breaking strength were measured successively.[11] Adhesions and fat tissue were removed from the segment after measuring anastomotic strength, and a 5-mm sample containing the suture line was collected and stored in liquid nitrogen for the hydroxyproline assay.

Anastomotic Collagen

Anastomotic samples were lyophilized, weighted, and pulverized. The hydroxyproline content, as a measure for collagen, was determined as described previously[12] in the 5-mm sample containing the suture line, essentially according to the method of Prockop and Udenfriend.[13]

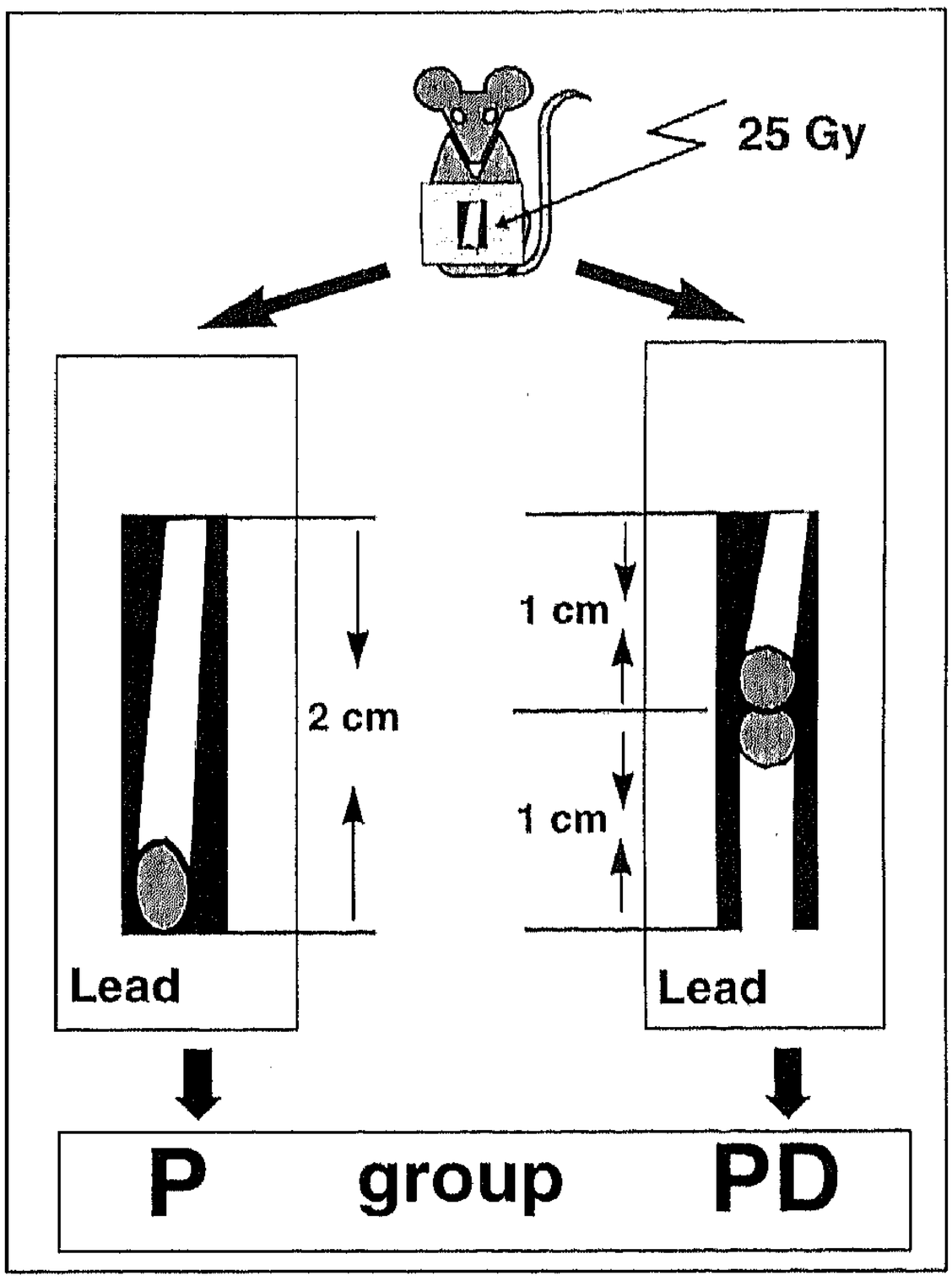

Figure 1. Experimental design of the study. Two experimental groups were studied. After resection of 1 cm of colon, rats were irradiated intraoperatively with a single dose of 25 Gy either to the proximal limb only (P group) or to both the proximal and distal limbs (PD group) of the colon before construction of an end-to-end anastomosis. In the P group, 2 cm of the proximal resection end was irradiated, and in the PD group, 1 cm of both the distal and proximal resection end was irradiated.

The percentage of acid-soluble hydroxyproline was assayed after suspending 10-mg powdered sample in 1.1 mL 0.5 mol/L acetic acid, incubating overnight at 4°C under rotation, and centrifuging for 1 hour at 42,000*g*. The supernatant was lyophilized and assayed for hydroxyproline. The pellet was resuspended in 1.1 mL 0.5 mol/L acetic acid containing 1 mg pepsin (Boehringer, Mannheim, Germany), incubating overnight at 4°C under rotation and centrifuging for 30 minutes at 13,000*g*. The resulting supernatant, containing the pepsin-soluble collagen, was treated as described above.

The collagen synthetic capacity in control and anastomotic tissues was quantitated in 5-mm tissue segments by measuring the incorporation of radiolabeled proline labeled with hydrogen 3 ([^{3}H]) into collagenase-digestible protein (CDP), according to a procedure validated before for rat intestinal tissue.[14]

In order to perform morphometric analysis of collagen, a 3-cm segment of the colon, including the anastomosis, was collected. The colon specimen was opened longitudinally, pinned on a plastic grid, and immersed in 4% buffered formalin (pH 7.4). The tissue was routinely processed and embedded in paraplast (Klinipath, Duiven, The Netherlands). Sections with a thickness of 4 μm were stained with hema-

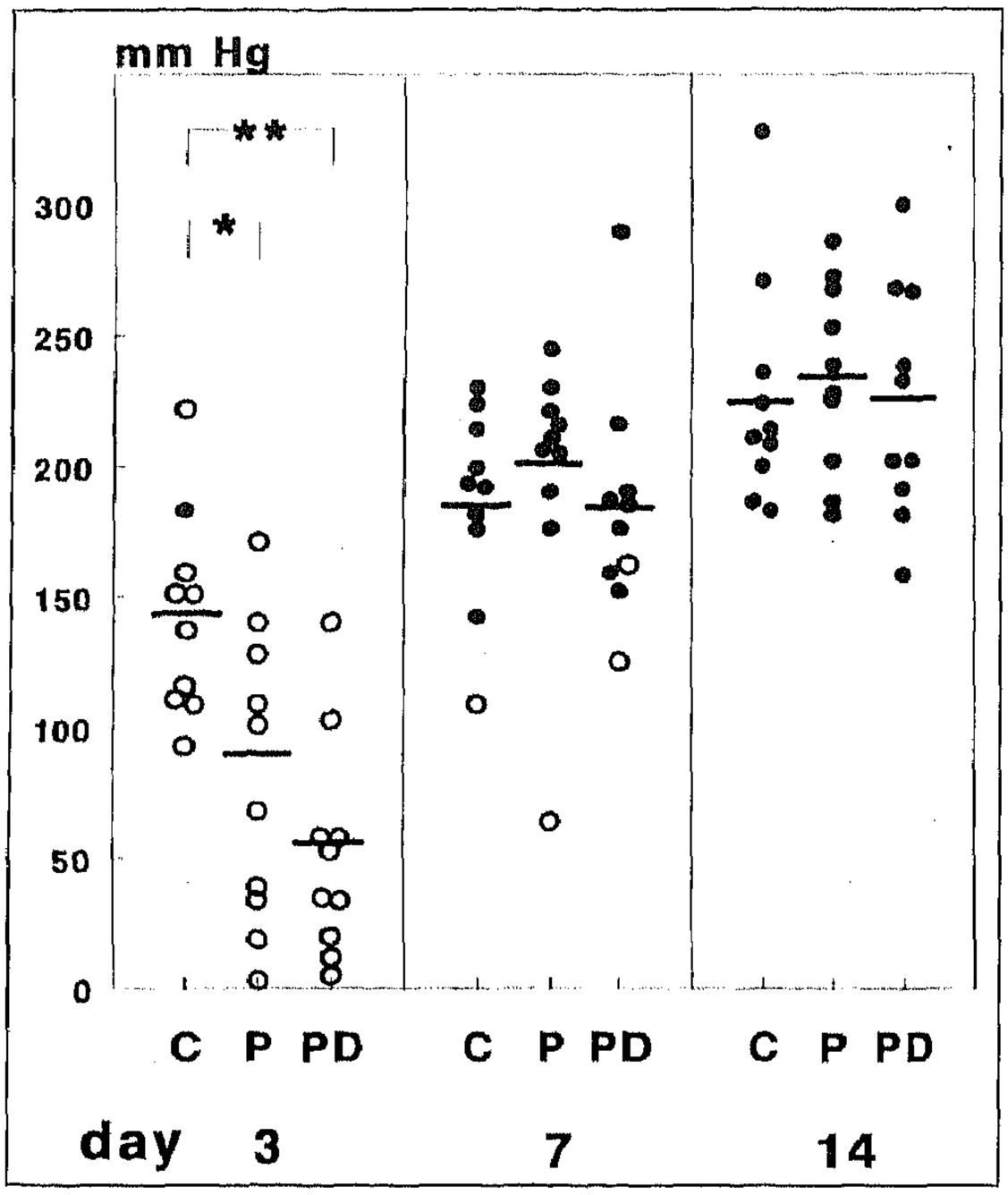

Figure 2. Anastomotic bursting pressure and bursting site. Each point represents a single anastomosis. The ○ are the bursting sites within the suture line. The ● are ruptures outside the anastomotic line. The **horizontal line** represents the average value. **P* <0.05; ***P* <0.01. C = control group; P = group with proximal resection edge of bowel irradiated; PD = group with both proximal and distal resection edges of bowel irradiated.

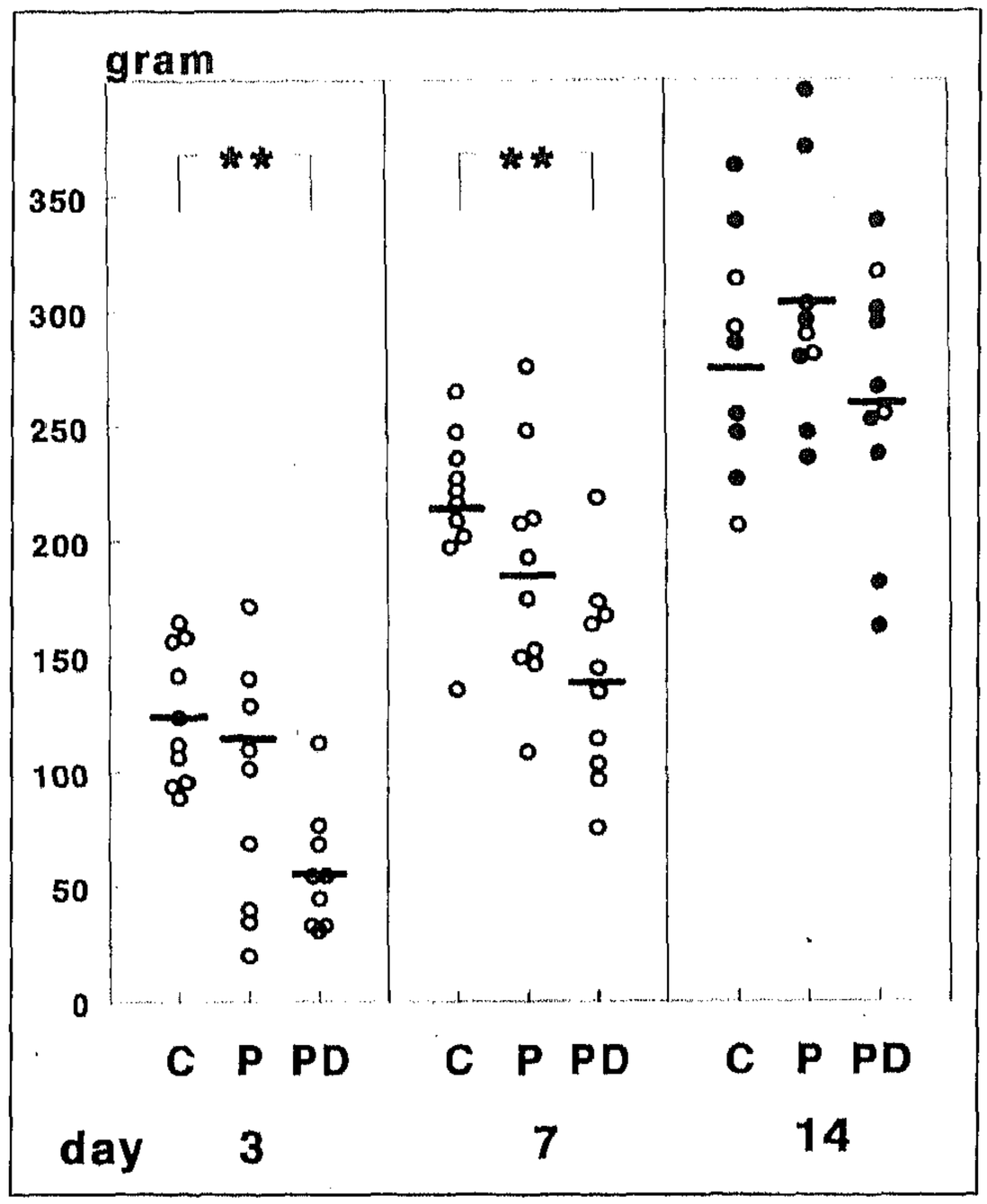

Figure 3. Anastomotic breaking strength and breaking site. Each point represents a single anastomosis. The ○ are breaking sites within the anastomotic line. The ● are ruptures outside the anastomotic line. The **horizontal line** represents the average value. ***P* <0.01. C = control group; P = group with proximal resection edge of bowel irradiated; PD = group with both proximal and distal resection edges of bowel irradiated.

toxylin-eosin or Sirius-red F3BA for collagen.[15] The collagen concentration in the actual wound area was analyzed by computer-aided morphometry, using a point counting program and a microscope with an M42 test grid. Measurements were taken directly from microscopic images (magnification ×400), calculating the volume fraction of collagen in the tissue as V(collagen)/V(tissue). V(collagen) was expressed in percentage of the wound tissue. For each specimen, 15 to 20 microscopic fields were chosen at random and measured.

Statistics

Group values were expressed as the mean ± standard deviation (SD). Differences between the experimental groups and the controls were tested for significance with a rank-sum two-sample test (Mann-Whitney *U* test), with significance levels set at 5%, 1%, and 0.1%.

RESULTS

All animals lost weight after the operation, but the loss was limited (data not shown). The day after operation the mean maximal weight loss was 8% of the weight before operation, and subsequently, a gradual increase in body weight was noted. The growth curves during the postoperative period were similar for the control group and both irradiated groups.

Anastomotic Strength

Previous experiments in our laboratory have shown that the average breaking strength of a colon anastomosis was unaffected by prior measurement of the bursting pressure. For instance, 3 days after operation the average anastomotic breaking strength of 10 anastomoses was 87 mm Hg (SD = 41), if measured directly, and 92 mm Hg (SD = 21) if measured after prior assay of the bursting pressure. Consequently, in the present study we performed both measurements on all anastomotic segments.

Individual and average values for bursting pressure and breaking strength of anastomoses, collected at 3, 7 and 14 days after operation in the 3 groups, are shown in **Figures 2** and 3, respectively.

In all groups, strength increased with time. Clearly, the average bursting pressure was lowered significantly 3 days after operation in the P group, whereas in the PD group both bursting pressure and breaking strength were strongly reduced with respect to the control group. With one exception in the control group, all disruptions were within the anastomotic area. After 7 and 14 days, no differences between the groups were found for the bursting pressure. At these time points, the bursting site was almost exclusively outside the suture line. At day 7, the breaking strength was significantly reduced in the PD group, but not significantly in the P group, although its mean value was 13% lower than in the control group. In

contrast to the bursting site, the breaking site was still located within the anastomoses in all groups. After 14 days, the breaking strength of both the P and PD groups was comparable to that in the control group, and the majority (67%) of the anastomotic segments disrupted outside the wound area. Thus, at this time point, the mechanical strength of both irradiated groups was restored to normal.

Anastomotic Collagen

As a measure for collagen, we first assayed hydroxyproline in the 5-mm segments containing the anastomosis. The average values for both hydroxyproline concentration and hydroxyproline content are shown in **Table I.** In all groups, both the concentration (expressed per mg wet weight) and the content (expressed per 0.5 cm anastomotic segment) increased from day 3 onwards. After 3 and 7 days, the hydroxyproline concentrations showed no variations between groups, but after 14 days the concentration was significantly increased in both irradiated groups when compared with the control group. If the hydroxyproline content was calculated, however, a significant reduction was observed on day 3 in the PD group. Comparison of the content at day 7 revealed no significant differences between the groups, although average values were lower in the irradiated groups. After 14 days, we found a significantly increased hydroxyproline content in both the P and PD groups when compared with the control group.

Successive extraction with acetic acid and pepsin solubilized a significant percentage of collagen from the anastomotic segments. For instance, at day 3 in the control group, 7.0% ± 1.8% (SD, n = 10) of total hydroxyproline was solubilized by acetic acid and 7.4% ± 3.3% by pepsin. Approximately 30% less hydroxyproline was extracted with acetic acid at day 7 or 14 than at day 3. Intraoperative radiation therapy did not affect the percentage of either acid-extracted or pepsin-extracted collagen.

Morphometric analysis was performed on Sirius-red–stained sections in order to obtain the collagen concentration in the actual wound area (**Figure 4**). This parameter is different from the hydroxyproline data presented above, because sampling of anastomotic segments for the purpose of biochemical hydroxyproline analysis is impossible without including tissue from the uninjured bowel wall. In the control group, the average collagen concentration doubled from day 3 to day 7. Thereafter, the average concentration remained stabilized around the level of day 7. In the P group, the average concentration also doubled from day 3 to day 7; however, the average concentration was lower at day 3 and even significantly lower at day 7 if compared with the control group. At day 14, values in the P group were similar to those found in the control group. In the PD group, the average concentration also increased from day 3 onwards. In comparison with the controls, however, the increase was much slower during the first week and much more pronounced during the second week. That resulted in a severe reduction in wound collagen volume at day 7, whereas at day 14 the average collagen volume percentage in the PD group had surpassed that in the control group.

The ex vivo collagen synthetic capacity of anastomotic tissue in the various groups at 3 and 7 days after operation is shown in **Table II.** In the control group, the anastomotic col-

TABLE I

Average Anastomotic Hydroxyproline Concentration and Content

Postoperative Day	N	Concentration (µg/mg Wet Weight)	N	Content (µg/Anastomosis)
Day 3				
Control group	10	9.4 ± 0.8	10	167 ± 26
P group	9	11.8 ± 1.4	9	159 ± 23
PD group	9	9.7 ± 1.1	9	124 ± 23*
Day 7				
Control group	10	11.9 ± 1.3	10	211 ± 53
P group	10	13.8 ± 2.5	10	184 ± 52
PD group	10	12.3 ± 1.6	10	177 ± 38
Day 14				
Control group	10	13.3 ± 0.9	10	217 ± 35
P group	10	15.3 ± 1.9†	10	320 ± 158*
PD group	10	15.7 ± 1.6†	10	458 ± 153‡

Data reported as average values ±SD.
**Significantly different from control, P <0.01.*
†Significantly different from control, P <0.05.
‡Significantly different from control, P <0.001.
P = proximal resection edge of bowel irradiated; PD = both proximal and distal resection edges of bowel irradiated.

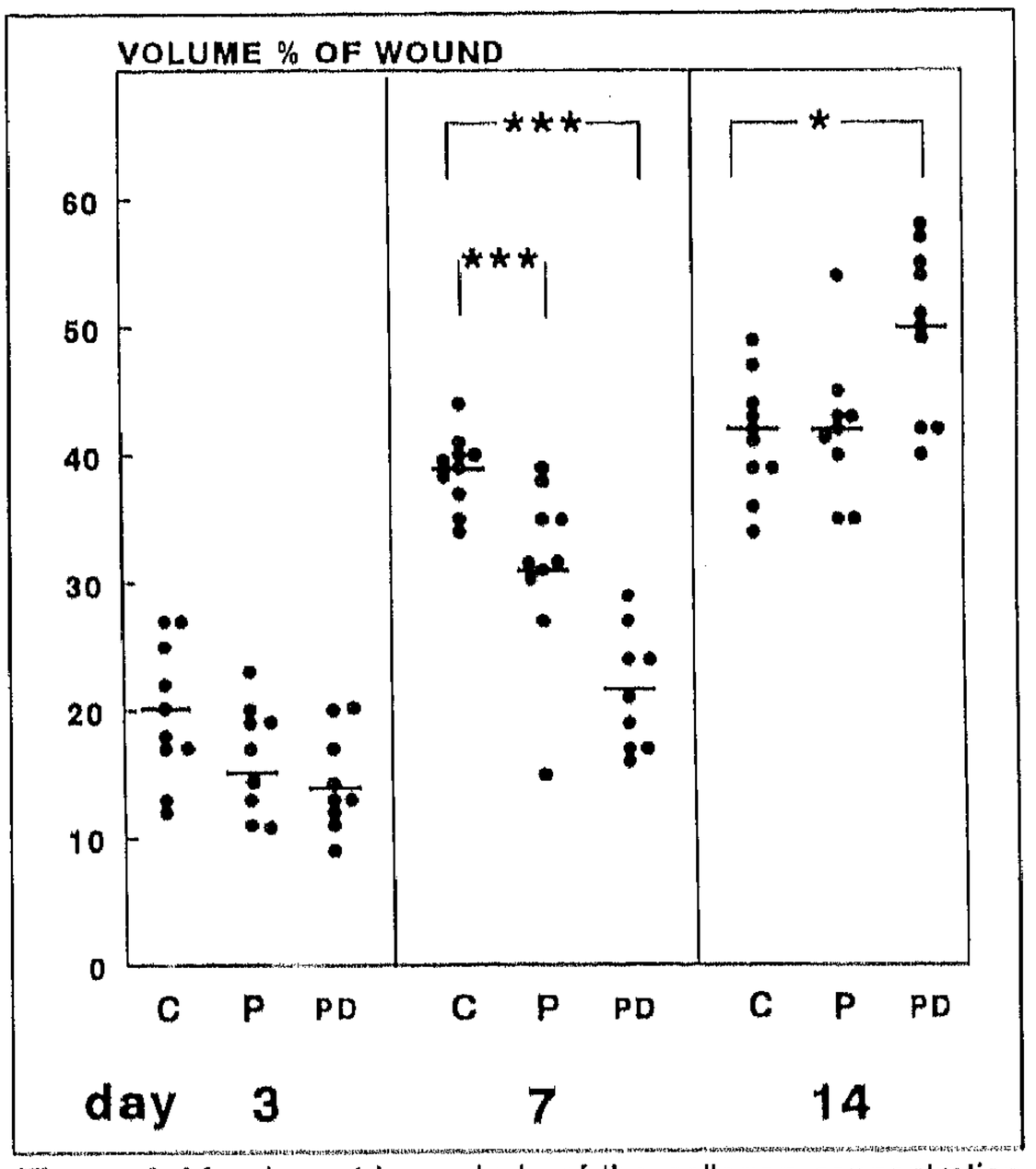

Figure 4. Morphometric analysis of the collagen concentration within the actual anastomotic area. Results from the individual anastomoses are given together with the average value (**horizontal line**). *P <0.05; ***P <0.001. C = control group; P = group with proximal resection edge of bowel irradiated; PD = group with both proximal and distal resection edges of bowel irradiated.

TABLE II

Ex Vivo Collagen Synthesis in Colonic Anastomotic Tissue

	N	DPM in CDP/ µG DNA	DPM in CDP/mg Wet Weight	DPM in CDP/ Anastomosis	% RCS
Uninjured colon	6	62 ± 10	218 ± 26	NA	0.60 ± 0.05
Postoperative day 3					
Control group	5	201 ± 66	772 ± 15	70,847 ± 22,775	1.67 ± 0.36
P group	6	226 ± 29	781 ± 95	53,029 ± 5,391*	1.73 ± 0.19
PD group	5	190 ± 33	499 ± 76*	33,664 ± 8,562*	1.50 ± 0.16
Postoperative day 7					
Control group	6	187 ± 45	640 ± 209	58,772 ± 14,436	2.00 ± 0.35
P group	6	178 ± 43	615 ± 148	46,533 ± 16,718	1.75 ± 0.36
PD group	6	229 ± 130	776 ± 317	42,093 ± 22,215	2.36 ± 0.59

Data represent average values ± standard deviation. Synthesis is expressed as radioactivity incorporated into collagenase-digestible protein (CDP) or as percentage relative collagen synthesis (RCS).
**Significantly different from control, P <0.05.*
DPM = Desintegrations per minute; NA = not applicable; P = proximal resection edge of bowel irradiated; PD = both proximal and distal resection edges of bowel irradiated.

lagen synthetic capacity increased significantly and specifically in comparison with uninjured intestine. At 3 days after operation, both absolute CDP and relative collagen synthesis were elevated approximately threefold. At this time, absolute collagen synthesis, expressed on the basis of DNA, wet weight, or protein (data not shown), was similar in the control and P groups. If the total synthetic capacity of the entire anastomotic segment was calculated, however, a significant reduction was seen in the P group. In the PD group, a more explicit reduction was observed. These effects were not specific for collagen, since the relative collagen synthesis remained unchanged. At 7 days after operation, no significant changes between the groups were observed, although the mean values for the CDP/anastomosis were lower in the irradiated groups.

COMMENTS

A high recurrence rate after resection for rectal carcinoma has stimulated investigations into adjuvant treatment. Radiation therapy has been frequently used as an adjuvant to surgery, since the combination of both is simple and straightforward.[1] Over the last 20 years, there has been an increasing interest in intraoperative radiation therapy for treatment of colon carcinoma.[2,4,16] Intraoperative radiation therapy may have its greatest clinical value in promoting local control of advanced tumors. Patients with T3, T4, and node-positive rectal adenocarcinomas frequently show local recurrences even after aggressive surgical resections. Preoperative or postoperative conventional external beam radiation therapy is routinely given to these patients, but an adequate dose cannot be reached because of the limited radiation tolerance of healthy abdominal tissues. Intraoperative radiation therapy then appears to be a valuable addition to the treatment protocol. Preoperative radiation induces tumor shrinkage and thus increases the surgical resectability of the tumor.[17,18] At surgery, intraoperative radiation therapy precisely directed to unresectable tumor or to areas of potential microscopic spread, or to both, will deliver an extra high dose of radiation that may improve the local control rate and the long-term survival.[17,19]

Although in clinical practice irradiation of an intestinal anastomosis will be avoided during intraoperative radiation therapy, irradiation of at least 1 anastomotic limb should be necessary in cases of low anterior tumor resections. We performed this study because information on the tolerance of normal colon tissue subjected to extensive surgical manipulation followed by a dose of irradiation and anastomotic construction is presently unavailable. For technical reasons, we chose to perform the study on intraperitoneal anastomoses. Since these are more protected and generally heal better than anastomoses below the peritoneal reflection, any adverse irradiation effects observed in intraperitoneal anastomoses will most probably be found to a greater extent in extraperitoneal anastomoses.

In both irradiated groups, healing is impaired during the first postoperative days as displayed by diminished anastomotic strength and reduced deposition of collagen. This finding suggests that tissues involved in anastomotic repair have suffered acute radiation damage. Furthermore, in the PD group, we found a more pronounced effect of irradiation than in the P group, suggesting that cells of both limbs are necessary for sufficient anastomotic repair. Therefore, in the clinical situation one limb of the anastomosis should be shielded from the radiation field in order to decrease the risk of excessive damage to wound-repair tissue.

It seems that the effects of intraoperative radiation therapy on the developing strength of colon anastomoses resemble those on the healing of small-bowel anastomoses. Saclarides et al[20,21] reported that a single dose of 20 Gy given intraoperatively to both limbs of the small bowel before continuity restoration impaired the healing of the anastomoses. If only one limb was irradiated, however, no difference with the controls was seen.[20] The authors suggested that anastomotic healing is only impaired when both limbs are irradiated. In contrast to the present study, however, only breaking strength was determined as a measure for anastomotic strength, and that only at 7 days after operation. We examined both bursting pressure and breaking strength, which were performed successively on the same anastomosis. These parameters together with those on collagen metabolism allow a more thorough evaluation of wound healing.[8] Furthermore, clinically detectable anastomotic leakage almost invariably occurs within a few days after surgery. Therefore, we also examined the extent of repair at day 3 after anastomotic construction. It is obvious that the most profound differences in anastomotic strength between both irradiated groups and the controls were observed at this particular time.

In this study, a single intraoperative dose of 25 Gy was given. Although this dose appears to be rather high in terms of human application, it has certainly been used in clinical practice.[22,23] We chose such a high dose for our initial studies in order to be able to better delineate the potential ef-

fects on the repair sequence. In further studies we will investigate how lower doses of intraoperative irradiation affect anastomotic repair.

Collagen is the major matrix protein that confers strength to the intestinal wall.[7,8] The significantly reduced collagen content and concentration in the anastomoses after intraoperative radiation therapy during the first week after operation, probably resulting from a lowered synthetic capacity, may explain the reduced wound strength. It may be that irradiation delays the migration and proliferation of fibroblasts to or into granulation tissue of irradiated bowel and thus causes a lower synthesis and deposition of anastomotic collagen. The fact that no differences in hydroxyproline solubility were found between the irradiation and control groups argues against insufficient collagen cross-linking as a cause of diminished strength.

Recent work in our laboratory has shown that irradiation of newly constructed colonic anastomoses does not affect early repair.[10] In that study, radiation was given after completion of the anastomosis. In the present study, intraoperative radiation therapy was given after resection but before anastomotic construction, leaving both limbs separated for at least 45 minutes between resection and construction. Apparently, radiation—in combination with the time interval—induces a delay in wound repair that may be related to changes in the acute phase of wound healing. Because of the treatment, blood supply may be reduced by changes in vascularity of the wound sites, resulting in a reduced fibrin deposition or a disturbed influx of polymorphonuclear and mononuclear cells in the wound area. These cells regulate collagen restoration, and anastomotic strength is largely dependent upon this process.

Although this study did not address long-term complications of intraoperative radiation therapy, the increased accumulation of anastomotic collagen in the irradiated groups at 14 days after operation suggests that fibrosis will gradually develop in the anastomoses constructed with irradiated intestinal limbs. The occurrence of fibrosis may lead to a reduction of the lumen and, eventually, to total obstruction. Therefore, a single radiation dose of 25 Gy or more may be above the tolerance dose for the large intestine. In a future study, we will investigate the long-term effects of intraoperative radiation therapy.

In summary, the results of this study show that intraoperative radiation therapy with a single dose of 25 Gy delays anastomotic repair in the rat colon. Although macroscopic anastomotic leakage was not observed in any of the rats, the wound strength was decreased in the irradiated groups, specifically in the PD group, during the first week after operation. This finding suggests that intraoperative radiation therapy may increase the risk of acute anastomotic failure.

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