Long-term Sequential Changes of Radiation Proctitis and Angiopathy in Rats

Hiroshi DOI1*, Norihiko KAMIKONYA1, Yasuhiro TAKADA1, Masayuki FUJIWARA1, Keita TSUBOI1, Hideharu MIURA1, Hiroyuki INOUE1, Masao TANOOKA1, Takeshi NAKAMURA2, Toshiyuki SHIKATA2, Takeshi KIMURA2, Tohru TSUJIMURA3 and Shozo HIROTA1

Radiation proctitis/Radiation colitis/Irradiation/Angiopathy/Rat.

The purpose of the present study was to establish an experimental rat model for late radiation proctitis, and to examine the assessment strategy for late radiation proctitis. A total of 57 Wistar rats were used. Forty-five of the rats were exposed to selective rectal irradiation with a single fraction of 25 Gy. These rats were sacrificed at the 4th, 12th, 24th, and 37th week following irradiation. The remaining 12 rats comprised the control group without irradiation. The rectal mucosa of each rat was evaluated macroscopically and pathologically. The number of vessels in the rectal mucosa was counted microscopically. In addition, the vascular stenosis was evaluated. In the results, the degree of clinical and macroscopic findings decreased following acute proctitis and developed later. In the pathological examination, mucosal changes and microangiopathy were followed up, as well. The absolute number of vessels in the rectum was the greatest at the 12th week following irradiation and was the lowest in the control group. The severity of the microangiopathy was also well evaluated. To conclude, we established an animal experimental model of late radiation proctitis, and also established an assessment strategy to evaluate objectively the severity of late radiation proctitis with focusing on microangiopathy using an animal experimental model. This model can be used as an animal experimental model of radiation-induced microangiopathy.

INTRODUCTION

Although radiation therapy is useful for the treatment of pelvic malignant tumors, such as uterine cervical cancer and prostate cancer, the occurrence of late rectal toxicity following irradiation (late radiation proctitis) is common. Radiation proctitis is a major clinical complication of pelvic irradiation, and has been reported to occur in 5% to 20% of the patients who have had radiation therapy in the pelvic region.1,1 The symptoms and signs that are associated with late radiation proctitis may appear from weeks to years after radiation, with an average range of 6 to 24 months before the appearance of symptoms.2,3 The symptoms of late radiation proctitis include rectal mucous discharge, diarrhea, urgency, pain, bleeding, fistula formation, and rectal stenosis.

Radiation proctitis has a prolonged course, and is histologically characterized by epithelial ulceration and marked stromal fibrosis. Moreover, it often induces rectal obstruction, and leads to a need for surgical resection.

Radiation induces a plethora of changes in the microvascular endothelium. These changes have been reported to play important roles in the mechanisms of the late radiation proctitis.4

Animal models of radiation proctitis have been reported in several studies.5–19 However, only a few studies have investigated both the long-term course and the histological changes using non-invasive techniques.5–8 In addition, there has been little research of the assessment strategies for late radiation proctitis.

In the previous study, we have reported a method to selectively irradiate the rectum without the use of surgical techniques and have also reported the efficacy of Polaprezinc against acute radiation-induced proctitis, using an experimental rat model.9,10

The purpose of the present study was to examine the
sequential changes of the rectum following irradiation and to establish an assessment strategy for late radiation proctitis in an experimental rat model.

**MATERIALS AND METHODS**

**Animals**

A total of 57 female Wistar rats, weighing 90–120 g and five weeks old were obtained from CLEA Japan Inc. (Tokyo, Japan). All of the rats were allowed to acclimate for seven days before the experiment. All of the animal procedures were approved by the Hyogo College of Medicine’s Institutional Animal Care and Use Committee prior to the initiation of the project.

Forty-five of the rats were irradiated. The follow-up time points were: 4 weeks, 12 weeks, 24 weeks, and 37 weeks following irradiation. The rats were divided into the following four groups according to the follow-up term:

- The 4w group: the rats were sacrificed 4 weeks following irradiation (n = 12).
- The 12w group: the rats were sacrificed 12 weeks following irradiation (n = 11).
- The 24w group: the rats were sacrificed 24 weeks following irradiation (n = 11).
- The 37w group: the rats were sacrificed 37 weeks following irradiation (n = 11).

In addition, the remaining 12 rats comprised the control group without irradiation (the RT (−) group).

The homogeneity of the body weights on the day of irradiation of the groups was confirmed using Bartlett’s test (P = 0.60).

**Irradiation**

The details of our animal model have been described previously.9) Each rat was anaesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and was weighed. Next, the three rats at a time were restrained in a vertical position and were taped by the tail to an acryl plate. Lead shielding was used to cover the rats except for a 2.5 cm long area of the lower pelvis, which contained the rectum in the middle of the field.

All of the rats were irradiated with a single X-ray fraction of 25 Gy, at 4 MV with a dose rate of 200 MU/min.

**Evaluation of the rectal changes**

Each rat was observed sequentially for clinical findings of proctitis, such as diarrhea and rectal bleeding, and the clinical findings were graded as follows: 0 = no symptoms; 2 = diarrhea; and 4 = gross bleeding. The irradiated rats in each group were sacrificed according to the experimental plan. The rats in the RT- group were sacrificed on the day equivalent of the tenth day following irradiation. In addition, the rectums in the RT (−) group were used as non-irradiated samples and examined regarding the absolute number of vessels.

The rectum of each rat was removed for the evaluation of the rectal changes immediately following sacrifice.

The macroscopic changes in the rectum were evaluated, and grading was conducted using the method previously described by Northway et al.10) The grading scores were as follows; 0 = normal mucosa; 1 = edema, mild hyperemia or decreased vascularity; 2 = diffuse hyperemia, multiple punctuate areas of hemorrhage or confluent areas of hemorrhages; 3 = the presence of erosions or frank hemorrhages; and 4 = ulcers.

The mild findings were defined as Grades 0 and 1, and the severe findings were defined as Grades 2 to 4.

The rectum was immediately fixed in 10% neutral buffered formalin solution. The fixed rectum was sectioned and was divided transversely into two or three equal segments (from the proximal rectum to the anus). Each segment was divided into two segments by the craniocaudal axis and was submitted for a histological analysis. In addition, each segment was made into two slides. Four specimens were examined pathologically for each rectum. All of the slides were stained with hematoxylin and eosin (H&E) and were examined using light microscopy. The length of the rectum that was examined for microangiopathy was 20 mm from the anus of each rat.

The epithelial findings were evaluated and were graded as follows; 0 = normal or minor alterations which could not be ascribed with certainly to radiation; 1 = slight crypt changes without a loss of the epithelium; 2 = crypt changes with a loss of the epithelium equaling less than half; 3 = crypt changes with a loss of the epithelium greater than half; 4 = a loss of the epithelium through the muscularis mucosa.

The absolute number of vessels was counted in the four specimens individually by microscopy. The sum of the numbers of the vessels in the four specimens was considered to be the number of vessels in the rat.

Angiographic criteria of arterial occlusive diseases was used as a reference for the evaluation of the stenosed vessels.20) The diameters of the lumen and the vascular endothelium were measured in the stenosed vessels, and the former was divided by the latter to evaluate the degree of the vascular stenosis (Fig. 1).

![Fig. 1. Evaluation of the stenosed vessels. The diameters of the lumen and of the vascular endothelium were measured, and the former was divided by the latter to evaluate the vascular stenosis.](image-url)
In addition, the degree of the vascular stenosis was graded as follows: 0 = no apparent stenosis; 1 = stenosis less than 50% (vessels with 1% to 49% stenosis); 2 = stenosis of more than 50% but of less than 75% (vessels with 50% to 74% stenosis); 3 = stenosis of more than 75% but that was not obstructive (vessels with 75% to 99% stenosis); and 4 = full obstruction.

The specimens of the rectums of the rats, which had been sacrificed at the 10th day following irradiation with the same protocol for administration in the previous study, were used in a pathological examination as a comparison group.

**Statistical analysis**

The results were presented as the mean ± standard deviation (SD). The results of the mucosal damages were compared, according to the mild or severe status, as applicable. The relationships between the groups were assessed using a chi-squared test, G-test and Fisher’s exact test. P values of less than 0.05 were considered to be statistically significant. Tukey’s all-pairwise-comparison test was used to identify the differences in the number of the vessels among the groups.

**RESULTS**

**Clinical follow-up and macroscopic findings**

The results of the clinical findings in the follow-up term are shown in Table 1. In addition, the following divided bar graph shows the proportion of the clinical findings at each follow-up term after irradiation (Fig. 2).

The clinical findings, which included diarrhea and melena, were found on the 10th day after irradiation. Subsequently, these findings decreased at the 4th week after irradiation, and developed again later in some rats.

One rat in the 4w group, one rat in the 12w group, and three rats in the 37w group died during the follow-up term. These five irradiated rats were excluded from the examination of the rectum, and the remaining 40 rats were eligible for the macroscopic and pathological examinations.

The mean body weights of the groups on the day when the rats were sacrificed were 200.93(+/– 10.00) g, 245.74(+/– 11.51) g, 266.17(+/– 13.44) g, 281.95(+/– 26.72) g, and 164.85(+/– 7.64) g in the 4w group, the 12w group, the 24w group, the 37w group, and the RT- group, respectively.

The results of the macroscopic findings are shown in Table 2. In addition, the following divided bar graph shows the proportion of the macroscopic findings in each group (Fig. 3). The macroscopic findings of the 12w group were significantly milder than those for the other groups.

<table>
<thead>
<tr>
<th>Table 1. Clinical findings during the follow-up terms</th>
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<tbody>
<tr>
<td>Group</td>
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<tr>
<td>RT (–)</td>
</tr>
<tr>
<td>4w</td>
</tr>
<tr>
<td>12w</td>
</tr>
<tr>
<td>24w</td>
</tr>
<tr>
<td>37w</td>
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</table>

4w = the 4w group, 12w = the 12w group, 24w = the 24w group, 37w = the 37w group. The numbers of rats with clinical findings are shown as Grade 0, Grade 2, or Grade 4 on the day following irradiation for each group.

Fig. 2. Clinical findings. The divided bar graph indicates the proportion of the clinical findings at each follow-up term after irradiation. No rats showed any perianal findings at the 12th week following irradiation. However, some of irradiated rats showed grade 2 of clinical findings after 24th week following irradiation.

<table>
<thead>
<tr>
<th>Table 2. Macroscopic findings</th>
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<tbody>
<tr>
<td>Group (number)</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>RT (–) (n = 12)</td>
</tr>
<tr>
<td>4w (n = 11)</td>
</tr>
<tr>
<td>12w (n = 10)</td>
</tr>
<tr>
<td>24w (n = 11)</td>
</tr>
<tr>
<td>37w (n = 8)</td>
</tr>
</tbody>
</table>

RT (–) = the RT (–) group, 4w = the 4w group, 12w = the 12w group, 24w = the 24w group, 37w = the 37w group. The 12w group showed a significantly milder result than the other groups.

Fig. 3. Macroscopic findings. The divided bar graph indicates the proportion of each grade in the macroscopic findings in each group. The severity of macroscopic findings decreased once and developed later.
Pathological findings

The sequential changes following irradiation are shown in Fig. 4.

In the pathological examination, crypt architectural distortion with the presence of inflammatory cells, planarization of the epithelium, and submucosal edema were found in the acute rectal injury on the 10th day following irradiation. In the 4w group, squamous metaplasia and submucosal fibrosis were observed in the rectal mucosa within the irradiated field. Vascular stenosis was observed with fibrinoid necrosis and a thickening of the vascular endothelium. In the RT (−) group, no apparent findings of mucosal changes or vascular stenosis were observed in the pathological examinations of all specimens.

The epithelial damages are shown in Table 3. In addition, the following divided bar graph shows the proportion of the epithelial damages in each group (Fig. 6). The proportion of rats with severe damage tended to increase after 24th week following irradiation. However, no significant differences were found regarding the severity of the damages among the groups.

After 12 weeks following irradiation, the glandular epi-

Fig. 4. Pathological findings of the epithelium in the irradiated rectums. Sequential changes of the rectal mucosa following irradiation are shown (H&E staining). (a) Rectal mucosa of a non-irradiated rat in the RT (−) group. (b) At the 10th day following irradiation; crypt architectural distortion with inflammatory cells, loss of the columnar shape, and the submucosal edema were observed. The epithelial findings corresponded to grade 2. (c) At the 4th week following irradiation (the 4w group); squamous metaplasia and submucosal fibrosis were observed in the rectal mucosa within the irradiated field. The epithelial findings corresponded to grade 1. (d) At the 12th week following irradiation (the 12w group); the regeneration of the damaged epithelium was found in the irradiated rectum. The epithelial findings corresponded to grade 1 in the epithelial damages. Whereas, the glandular epithelium was displaced into the submucosa (heterotopia). (e) At the 24th week following irradiation (the 24w group); the findings of grade 4 in the epithelial damages were observed in three rats. The loss of epithelium reached submucosa. (f) At the 37th week following irradiation (the 37w group); the regeneration of the damaged epithelium proceeded. The epithelial findings corresponded to grade 1. While, the heteromorphic structure remained.

Table 3. Epithelial damages of the pathological examination

<table>
<thead>
<tr>
<th>Group (number)</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4w (n = 11)</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>12w (n = 10)</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>24w (n = 11)</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>37w (n = 8)</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

4w = the 4w group, 12w = the 12w group, 24w = the 24w group, 37w = the 37w group. There were no significant differences among the groups.

Fig. 5. Epithelial damages. The divided bar graph indicates the proportion of each grade in the epithelial damages. The severity of macroscopic findings decreased once and developed later, although there were no significant differences among the groups.
thelium was displaced into the submucosa. In addition, regeneration of the damaged epithelium and a heteromorphic structure were observed in the irradiated rectum (heterotopia). The cells lining the herniating glands exhibited remarkable structural atypia, whereas nuclear atypicality was not apparent in these cells. In addition, these findings evolved over time and later caused lumenal stenosis. Meanwhile, the squamous metaplasia in the epithelium had disappeared at the 12th week after irradiation.

The proportion of rats with squamous metaplasia was 72.3%, 0.0%, 27.3%, and 14.3% in the 4w group, the 12w group, the 24w group, and the 37w group respectively. In addition, significant differences in these proportions were observed between the 4w group and the other groups (P < 0.05).

The proportion of rats with heterotopia was 9.1%, 70.0%, 63.6%, and 100.0% in the 4w group, the 12w group, the 24w group, and the 37w group respectively. In addition, significant differences were observed in these proportions between the 4w group and the other groups (P < 0.05).

**Angiopathy**

Strictures with a thickening of the vascular endothelium were observed in all of the irradiated groups. The vascular changes consisted of endothelial thickening and arteriolar sclerosis. The angiopathy was observed selectively in the arteries. In contrast, no apparent vascular stenosis was present in the veins. A narrowing lumen was observed in some rats with the acute proctitis on the 10th day following irradiation. In addition, the vascular endothelial damage was mainly due to nuclear bulging in this group. However, the thickening that accompanied the fibrinoid necrosis in the 4w group, and the thickening of endothelial lining were significant later. The differences of the endothelial changes in the microvessels between the acute proctitis and the late proctitis are shown in the Fig. 6.

The absolute number of vessels per individual was 289.7 (±63.5), 385.8 (±60.6), 256.6 (±70.0), 282.1 (±57.1), and 141.4 (±47.5) in the 4w group, the 12w group, the 24w group, the 37w group, and the RT- group, respectively. In addition, significant differences were observed between the 12w group and the other groups, and between the RT- group and the irradiated rats of the other groups (P < 0.05).

The degree of stenosis was evaluated in the microvessels microscopically. The number of stenosed vessels in each group is shown in Table 4. In addition, the following divided bar graph shows the proportion of each grade in all stenosed vessels (Fig. 7). No significant differences were found among the groups in terms of the proportions of severe vascular stenosis.

![Fig. 6.](image)

**Table 4.** The number of stenosed vessels per individual

<table>
<thead>
<tr>
<th>Group (number)</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4w</td>
<td>17.3</td>
<td>5.6</td>
<td>12.4</td>
<td>11.2</td>
</tr>
<tr>
<td>12w</td>
<td>14.6</td>
<td>7.6</td>
<td>9.5</td>
<td>9.3</td>
</tr>
<tr>
<td>24w</td>
<td>16.7</td>
<td>6.5</td>
<td>6.0</td>
<td>4.8</td>
</tr>
<tr>
<td>37w</td>
<td>22.7</td>
<td>5.0</td>
<td>7.0</td>
<td>6.4</td>
</tr>
</tbody>
</table>

4w = the 4w group, 12w = the 12w group, 24w = the 24w group, 37w = the 37w group. The severity of the stenosed microvessels was evaluated. No significant differences were found among the groups regarding the proportions of severe vascular stenosis.

![Fig. 7.](image)
The proportions of the stenosed vessels that occupied a portion of the absolute number of the vessels were 16.0%, 10.6%, 13.3%, and 14.6% in the 4w group, the 12w group, the 24w group, and the 37w group, respectively. In addition, no significant differences were observed between the 4w group and the 37w group, or between the 24w group and the 37w group (P < 0.05).

**DISCUSSION**

Late radiation proctitis commonly occurs as a result of radiotherapy for pelvic malignancies. However, no recommended standard treatment exists, and current management is often unsatisfactory.

Hubmann originally described a method to irradiate the rectum of the rat without the use of surgical procedures and with minimal radiation exposure to the other organs. This technique has been used in several reports, and acute radiation proctitis has been described in the previous studies. 6–12) Previously, we have confirmed that this animal model allowed for selective irradiation to the rectum, and have reported the efficacy of Polaprezinc for acute radiation proctitis using the animal experimental model. 9)

In the present study, the sequential changes following irradiation were observed in the rectal mucosa of irradiated rats. In the clinical and macroscopic findings, the results that were greater than grade 2 findings were not found at the 12th week following irradiation, but were found after 24 weeks of follow-up following the irradiation. The results of the clinical and macroscopic findings indicated that late radiation proctitis followed the acute toxicity after irradiation. In addition, the 12th week following irradiation seemed to be a transitional period from acute toxicity to late toxicity. Whereas, the clinical findings correspond to grade 2 were found only in the 4w group at the 4th week following irradiation. Defecation status or technical error might cause these results of the clinical findings. Furthermore, the severity of the macroscopic findings increased after 24 weeks of follow-up following irradiation in contrast to the epithelial damages of the pathological examination. The epithelial damages of the pathological examination were evaluated only regarding the degree of the loss of epithelium, however the angiogenesis or heterotopic structures existed. The differences between the macroscopic findings and the pathological examination might be caused by the feature of our method to evaluate the rectal mucosa. Therefore, the rectum should be examined clinically, macroscopically, and pathologically, to be evaluated with more precision from various perspectives.

Heterotopia was observed in the irradiated rectum after 12 weeks of follow-up following the irradiation. In addition, no significant differences in the frequency of heterotopia were observed among the three groups. These findings correspond to the pathological findings of proctitis cystica profunda in previous reports. 21–23)

Proctitis cystica profunda (PCP) is an uncommon entity, and its exact pathogenesis remains obscure. PCP has been reported as a late complication of pelvic irradiation in man, and it usually occurs when more than a decade has elapsed between the completion of the radiotherapy and the clinical emergence. 21–23) In addition, the adenocarcinoma that was associated with colitis cystic profunda had been reported in previous reports. 24–26) The incidence of PCP was 70.0% in the rats with 12 weeks of follow-up following irradiation. Whereas, we have reported that the incidence of ulceration was 42.9% during the acute phase (at the 10th day following irradiation) in the previous study. 9) PCP is likely to be recognized most often in solitary rectal ulcer syndrome. However, the difference suggests the ulceration during the acute phase may not be related to the occurrence of PCP.

Fajardo et al. have reported radiation-induced arteriolar vasculitis on coronary arteries and iliac arteries using minipigs. 27) Acute arteriolar vasculitis with fibrinoid necrosis was revealed 28 days after irradiation in this previous study. In the present study, endothelial thickening with fibrinoid necrosis was observed four weeks following irradiation. This result is consistent with the results of the previous report. Menendez et al. have investigated the effect of irradiating the abdominal aorta in rats. 28) These authors also reported the effects of radiation on endothelial function. The pathological results of vascular stenosis in the present study corresponded to those of this previous report.

The sequential changes of radiation-induced microangiopathy were examined in the present study. Radiation induced angiopathy was observed selectively in arteries. In addition, the angiopathy mainly consisted of a thickening of the endothelium. The fibrinoid necrosis of the vessel walls causes the proliferation of the endothelial lining, which results in luminal narrowing, and can lead to ischemia. In addition, fibrinoid necrosis was observed after four weeks following irradiation. In many patients, the hematochezia is most severe 18 to 24 months after radiation therapy. The presence of the telangiectatic vessels causes to bleed easily. Therefore, the severity of hemorrhagic radiation proctitis is often due to the elevation of the friable rectal mucosa with the telangiectatic vessels. In the present study, the number of vessels was the greatest during the 12 weeks following irradiation. The results of the evaluation of the angiopathy at this time point seem to be reflected in the fragile vessels in the hemorrhagic late radiation proctitis. Whereas, the numbers of stenosed vessels per individual were not different among groups. In addition, the results in the clinical and macroscopic findings were greater after 24 weeks of follow-up following the irradiation. Radiation-induced vascular stenosis seemed to cause the angiogenesis followed by late radiation proctitis and to increase the number of functional vessels.

The diameter stenosis has been used widely to evaluate
the vascular stenosis in the clinical practice, and has also been used in the basic studies, although the area stenosis and the blood flow velocity seem to correlate better with the hemodynamic effect than the diameter stenosis.\textsuperscript{20,29–31} In addition, the area stenosis of the microvessels may be difficult to examine in rats. Therefore, we propose that the measurement of the diameter stenosis is useful to evaluate the microangiopathy. Furthermore, we increase the number of examined specimens to four in each rectum of all rats to clear the artifact of the tortuosity, and to evaluate homogeneously. As the result, this assessment strategy seems valuable to compare the severity of the microangiopathy among the groups. However, the relationship between the microangiopathy and the decreased arterial perfusion in the rectum should be investigated in the future study.

Ulceration, rectal obstruction, and carcinogenesis in the rat intestine have been reported as late adverse effects after three to fourteen months in a long-term observation after irradiation.\textsuperscript{4,7,14–19} Black et al. have described a method to evaluate a radiation-induced small intestinal disorder using a grading system.\textsuperscript{19} Although, the findings of the epithelium were unifying, individual differences were apparent. In addition, this method has less objectivity. Microangiopathy, which consists of vascular stenosis and angiogenesis, seems to impact the frequency and the severity of late radiation proctitis. We consider that our method, which focuses not only on epithelium but also on microangiopathy, is to be useful to evaluate the severity of late radiation proctitis.

In the present study, we examined the changes in the rectal mucosa systematically using objective assessment strategies of radiation induced microangiopathy in an animal experimental model, and suggested that this method was useful for evaluation of late radiation proctitis. To the best of our knowledge, no studies have been published regarding the same assessment strategy of radiation proctitis. In addition, we consider this rat model can be applied to an animal experimental model of radiation induced microangiopathy.

We have described the efficacy of Polaprezinc, an anti-ulcer drug, and its anti-inflammatory effect for acute radiation-induced rectal disorders, using an experimental rat model.\textsuperscript{8} Denham et al. and Heemsbergen et al. have reported that acute injury often contributes to the development of chronic changes in the rectum.\textsuperscript{32,33} The suppression of radiation-induced acute injury and inflammation may decrease the occurrence and severity of late radiation proctitis. We are investigating the biochemical and molecular biological changes following irradiation, and the efficacy of pharmacological approaches to suppress late rectal toxicity after irradiation is presently under investigation.

In conclusion, the sequential changes of rectal mucosa and arteries were examined particularly and systematically. We established an animal experimental model of late radiation proctitis, and also established an assessment strategy to evaluate the severity of late radiation proctitis, with a focus on microangiopathy.

\section*{ACKNOWLEDGEMENTS}

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