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Prolonged Radiation Damage in Rat Colon and Urokinase Expression in Epithelium

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Although radiation therapy plays important role in the treatment of gynecological tumors, it may cause radiation injury as a late effect. Several recent reports show that urokinase such as urokinase type plasminogen activator (uPA) contributes to the repair of ulcerative lesions of the colon epithelium. We studied radiation induced enterocolitis using rat animal models. Seventy-two female Wistar rats were irradiated by a single fraction dose of 36Gy at laparotomy. Histological changes and activity of urokinase system were investigated after irradiation. Ulcers were observed in irradiated field in 12 of 19 animals (63%) even at 60th week after irradiation. Urokinase expressions were observed in the margins of active ulcer. Urokinase was thought to play important role in exacerbation of ulcer formation. Expression of uPA was also observed in submucosal glands. Ischaemic changes were not observed in irradiated colon despite sclerosing vasculitis. It is suggested that uPA played reciprocal roles in radiation induced enterocolitis: healing and aggravation of ulcer.

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Key Words: rat, radiation induced enterocolitis, epithelial reproduction, uPA

Abbreviations: uPA; urokinase type plasminogen activator, uPAR; urokinase type plasminogen activator receptor, tPA; tissue type plasminogen activator, RIS; radiation injury score

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Introduction

While radiation therapy is considered useful in the treatment of gynecological malignant tumors such as uterine cervical cancer, the occurrence of late radiation damage is not negligible. Radiation induced enterocolitis occurs in about 10% of the patients who had radiation therapy to the pelvic region. Radiation induced enterocolitis occurs in the rectosigmoid colon and small intestine within the irradiated area several years after irradiation. It has a prolonged course, and is histologically characterized by epithelial ulceration and marked stromal fibrosis (1)(2). Moreover, it often induces intestinal obstruction, and needs surgical resection (1).

In several reports, radiation induced enterocolitis may cause secondary adenocarcinoma of the colon (3)(4)(5). Radiation induced enterocolitis is characterized by prolonged clinical course and also by a latent period after irradiation, unlike colitis of other types.

Walsh reported the first case of radiation damage in human colon in 1897. Black (1), Hubmann (2) and others have reported animal models of radiation induced enterocolitis, and investigated the correlation between radiation dose and the incidence of radiation induced enterocolitis as well as its outcome. Geisinger quantitated histological findings using radiation injury score (RIS), and studied the correlation among RIS, radiation dose and fraction. However, there have been only a few studies that investigated both the long term course and histological changes.

Urokinase (urokinase type plasminogen activator; uPA and tissue type plasminogen activator; tPA) is considered as one of the chemical mediators that is involved in the regeneration of the damaged epithelial cells of the colon and in the invasion of tumor cells (7). Urokinase is one of the proteinases that is produced in and secreted by the epithelial and mast cells. Urokinase makes complex with its receptor uPAR on tissue surface, and activates other proteinase

precursors, such as plasmin and metalloproteinase (8).

We investigated the mechanism of radiation induced enterocolitis. Our study focussed on the evaluation of long term histological changes, particularly on the relationship between urokinase and depth of the ulcer.

MATERIALS AND METHODS

Animals

A total of 77 female Wistar rats (Kyudou Company, Japan) were used in the experiment. These animals were irradiated at approximately five weeks of age and weight 90-150g. They were housed three per cage, and fed with laboratory-pelleted formula and tap water *ad libitum*. Food was removed one day before irradiation but water was available. Each animal was anesthetized with an intraperitoneal injection of sodium pentobarbital (32.5-48.0 mg/kg).

After a mid-abdominal laparotomy, the animal was placed on a wooden platform midway between two rectangular blocks that ran parallel to the long axis of the rat's body in supine position. Small sheets of lead (2mm thickness) were placed lateral to the colon between the X-ray source and the abdominal viscera as a shield from radiation effects. Only the descending colon was irradiated using radiation field of 10 by 26mm. Radiation field was kept moist with physiologic saline-soaked gauze pads. Irradiation was given using an EXS-300 X-ray machine (TOSHIBA Co., Japan). Total radiation dose was 36Gy by single fraction (200kV voltage, 15mA current, 0.5mm aluminum filter, dose rate 0.875Gy/ min). After the irradiation, the animals were sutured and housed in the same way as before irradiation. All animals were carefully observed every day, and weighed twice per week in the first month after the irradiation, and twice per month in the second month. When abdominal distention, a sign of intestinal obstruction, continued or the body weight reduced, the animals were killed before the general condition deteriorated. The animals were sacrificed at ten weeks intervals from 20th week to 60th week after irradiation. Five animals were sham irradiated, and housed in the same conditions as irradiated rats, and sacrificed at 60th weeks after laparotomy.

Assessment of histopathologic injury

Animals were laparotomied and inspected for macroscopic pathologic alterations. The specimens were fixed in neutral buffered formalin, processed, embedded, and blocked in paraffin. Sections were cut at three micrometers thickness, and stained with hematoxylin and eosin.

Investigations of the histological changes were conducted on the following items.

(1) Radiation Injury Score (RIS), (2) microscopic findings, (3) expression of uPA and uPAR.

(1) Radiation Injury Score (RIS) is a method of evaluation reported by Hauer and Jensen that indicates the severity of radiation intestinal injury (9). For the colon of each rat, one macroscopic and seven microscopic alterations were scored and the sums of these eight individual scores were used to evaluate the severity of radiation injury. RIS, varying from 0 to a maximum possible score of 22 (Table.1), correlates well with actual intestinal radiation damage (10). To evaluate deep ulcer formation often seen in radiation induced enterocolitis that is not properly evaluated by RIS, we examined vertical extension of the ulcer. Epithelial ulcer was classified in terms of both morphology and depth. Open ulcer was defined as the ulcer associated with loss of superficial reproductive epithelial cells and ulcer scar was defined as the ulcer covered by regenerated epithelium. The depth of ulcer was classified into Ul-1 to Ul-4. (Ul-1: epithelial erosion, Ul-2: penetrating through muscularis mucosa, Ul-3: penetrating into propria mucularis propria, Ul-4: penetrating through mucularis propria). Infiltration of inflammatory cells was divided into three classes according to

Table 1. Point values for individual pathologic alterations, which make up total radiation injury, score (RIS)

Macroscopic alterations of irradiated intestine
1. Pale-gray serosa
2. Palpable thickening of intestinal wall
3. Stenosis with proximal dilatation
Histopathologic alterations of irradiated intestine
Mucosal ulceration
1. Small, focal ulceration
2. Multiple foci of ulceration
3. Ulceration involving more than half of the irradiated intestinal segment
Epithelial atypia
1. Abnormally-oriented crypts or abnormally-shaped villi
2. Irregular crypt regeneration with atypical epithelial cells
3. Adenocarcinoma
Thickening of serosa
1. Slight thickening of subserosa; hyperplasia of peritoneal mesothelium
2. Marked thickening of subserosa
3. Extreme thickening and fibrosis of subserosa
Vascular sclerosis
1. Slight thickening and hyalinization of vessel wall
2. Vessel wall double normal thickness; hyalinization and sclerosis
3. Extreme sclerosis with marked stenosis or complete obliteration; fibrinoid necrosis.
Fibrosis of intestinal wall
1. Submucosa double normal thickness; broadened and hyalinized collagen fibers
2. Submucosa three-to-four times normal thickness; abnormal collagen fibers
3. Massive fibrosis including muscularis
Iletis cyctica profunda
1. Submucosal glandular inclusions
2. Submucosal cysts with polypoid elevation of the mucosa
3. Large cysts extending into the muscularis
Lymph congestion
1. Dilated lymph vessels or cystic collections of lymph
0 = no injury, maximal total score = 22 (Poulakos et. al. 1990)(1

the density of infiltration (-; no infiltration +; mild infiltration, ++; severe infiltration). Micro abscess that consisted of submucosal infiltration of inflammatory cells having no relation with epithelial ulcer was not included in the evaluation of inflammatory cell infiltration.

Immunohistochemistry and image analysis

Evaluation of urokinase system (uPA, tPA, and uPAR) was conducted by the technique of immunohistochemical staining of the specimens. Each specimen was routinely processed in paraffin wax, sectioned at three micrometers thickness. After deparaffinised sections, non specific protein binding was reduced by incubation in blocking solution (DAKO protein block, serum free) for 10 minutes at 37℃. A goat monoclonal anti-uPA antibody (sc6831, Santa Cruz Biotechnology, Inc. CA, U.S.A), tissue type plasminogen activator (tPA) antibody (sc5239) and anti-uPAR antibody (sc9795) were diluted in PBS buffer (each 1: 50) and applied to the sections for one hour at 37℃. The secondary biotinylated goat anti-rabbit antibody (1:200, anti-goat IgG biotin, Santa Cruz Biotechnology, Inc.) was applied for 30 minutes at 37℃. For blocking endogenous peroxidase activity, the specimens were incubated in 0.3 % hydrogen peroxidase-methanol solution for 10 minutes. After removing non-bound antibody, the preformed avidin-biotin peroxidase complex was applied for 20 minutes at 37℃. (LSAB-kit, DAKO, Japan). Subsequently, the slides were developed to visualize with diaminobenzidine tetrahydrochloride solution (containing imidazol 13.4 mg and 0.01 % hydrogen peroxide). The control slides as well as other slides were stained without applying primary antibody. The number of uPA expressed cells was counted under the high magnification (X250). The number of cells in a horizontal margin beside ulcer was calculated and their density per micrometer was computed. It was divided into three classes according to immunostained cell numbers in one low power visible field (-; <10% positive cell number, +; 10-70%, ++; >70%).

Statistical assessment was done using by Stat View 5.0 (HULINKS Inc., Japan), by Fisher's protected least significant difference.

RESULTS

(1) Natural course and macroscopic change after irradiation

15 of 72 irradiated animals (29.2%) died during the follow up period. Additional six animals were sacrificed because of deteriorating clinical status. The remaining 51 animals were sacrificed: six animals each at 20th, 30th and 40th week, 14 at 50th week, and 19 at 60th week. None of the five sham irradiated animals died. Body weight was not significantly different between the irradiated animals and sham irradiated animals. However body weight of the animals that died after 40 weeks after irradiation, was significantly different ($p<0.05$) from surviving and sham animals (Fig.1). At macroscopic observation of the 51 animals sacrificed at scheduled weeks, colonic stenosis was observed in 13 animals, wall thickening of the colon in nine, and irregular mucosa and ecchymosis of the mucosa in six. Seven of 13 colonic stenosis were accompanied by intestinal dilatation of oral side. Polypoid lesions were observed in one animal each sacrificed at 40th and 60th weeks, and their pathological diagnosis were mucinous adenocarcinoma. Tumor lesions of the small intestine were observed in two animals of 50th week group. These tumors, invasive mucinous adenocarcinoma, were located distal to the ligament of Treitz. One of them was accompanied by axillary lymphnode metastasis. One of the five sham irradiated animals revealed mild stenosis of the colon, and two showed intestinal wall thickening and irregularity of the colon.

(2) Histological changes

Radiation damage was observed in the epithelium and stroma within the irradiated field of the colon. Epithelial ulcers, atypical epithelium and epithelial

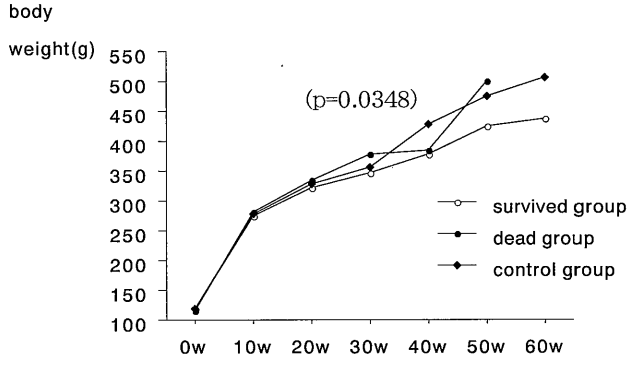

Figure 1. Course of body weight after irradiation

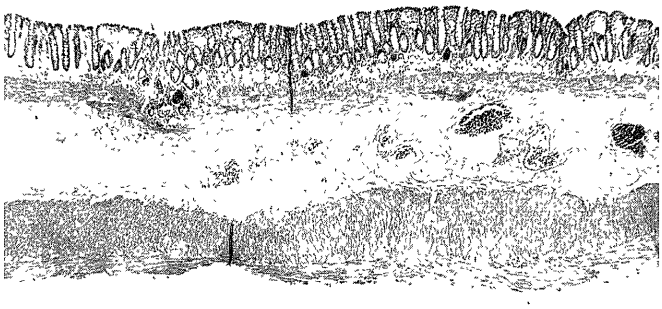

Figure 2. Radiation colitis with repaired ulcer at 50th week. The depth of ulcer was thought to Ul-2. Moderate thickening of muscular mucosa and submucosa was observed. Arterial wall thickening was severe, however, no finding of ischemic colitis was observed. Total RIS of the colon was calculated to seven (X10).

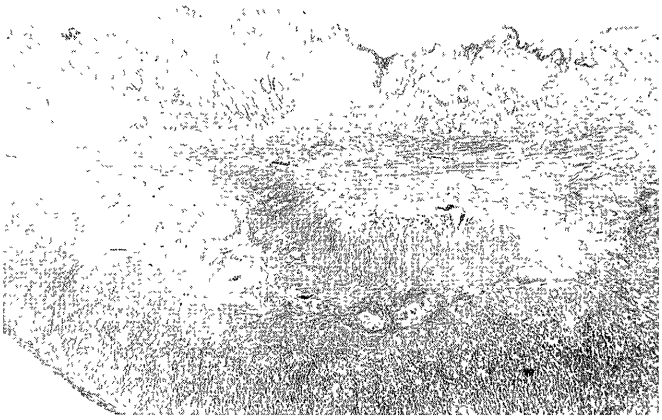

Figure 3. Radiation colitis with open ulcer at 30th week. Open ulcers and atrophic epithelium were observed. Muscular mucosa was destroyed, and stroma seemed markedly thickening with fibrous change. Epithelial cells and cystic dilatation of glands were seen in submucosa. The total RIS was calculated to 21 (X10).

Table 2. The number of the ulcers; Ul-0 means no definite ulcer formation.

*()incidence(%)

	20w	30w	40w	50w	60w	Total
no ulcer	2 (33.3)	0 (0 0)	1 (16.6)	1 (7 1)	7 (36.8)	11
Ul-2	1 (16.6)	1 (16 6)	1 (16 6)	1 (7 1)	6 (31.6)	10
Ul-3	2 (33.3)	3 (50 0)	2 (33 3)	7 (50.0)	4 (21.1)	18
Ul-4	1 (16 6)	2 (33.3)	2 (33.3)	5 (35.7)	2 (10.5)	12
Total	6	6	6	14	19	51

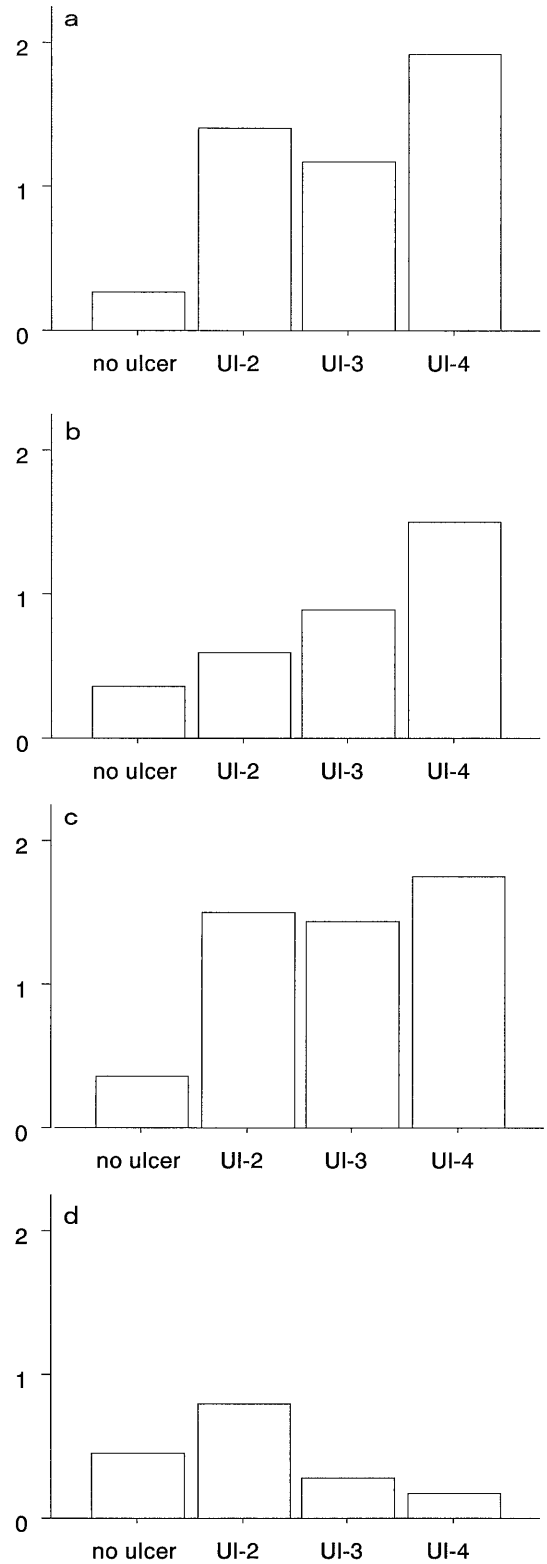

Figure 4. The average density of infiltration. (a: total cells of infiltration, b: eosinophil, c: neutrophil, and d: lymphocyte) Marked neutrophilic infiltration was observed regardless of the depth of ulcers, but the eosinophilic infiltration was observed in shallow ulcers. On the contrary, lymphocytic infiltration was scanty in deep ulcers

glands under submucosa were regarded as epithelial radiation damages. Epithelial ulcers were observed in 40 of the 51 animals (78.4%). 35 of the 40 ulcers showed progression over muscularis mucosa with loss of overlying epithelium and the remaining five (12.5%) were scar of ulcer with repaired epithelium (Fig. 2, 3.). Of all 40 ulcers, there were ten (19.6%) Ul-2 ulcers, 18 (35.3%) Ul-3 ulcers, and 12 (23.5%) Ul-4 ulcers (Table 2.). There was no significant correlation between the depth of ulcer and the time from irradiation. Infiltration of inflammatory cells, that indicates the activity of ulcer, was observed surrounding the ulcers. In all ulcers, more infiltration of inflammatory cells was observed with progression of the depth of ulcer. Marked neutrophilic infiltration was observed regardless of the depth of ulcer, but eosinophilic infiltration was observed in superficial ulcers. On the contrary, lymphocytic infiltration was scantly in deep ulcers (Fig. 4a-d). Regenerative mucosa was often observed in the glands around the ulcers, and two or more RIS were present in 27 (52.9%) cases. Epithelial glands under submucosa were observed in 23 of 51 animals (45.1%) (Fig. 5.) (Table 3.): in nine of 11 Ul-4 ulcers (81.8%), 14 of 18 Ul-3 ulcers (77.8%), and two of 11 Ul-2 ulcers (18.2%). While epithelial glands under submucosa were observed in developed ulcers over muscular mucosa frequently, it was observed in three of 11 animals (27.3%) that had no ulcer. Epithelial glands under submucosa were observed in each five of the six animals (83.3%) in each of the 20th, 30th, and 40th week, nine of 14 (64.3%) in 50th week, and four of 19 (21.1%) in 60th week (Table 4.).

Fibrosis and vascular changes were observed in all animals to varying degrees. Lymph duct congestion

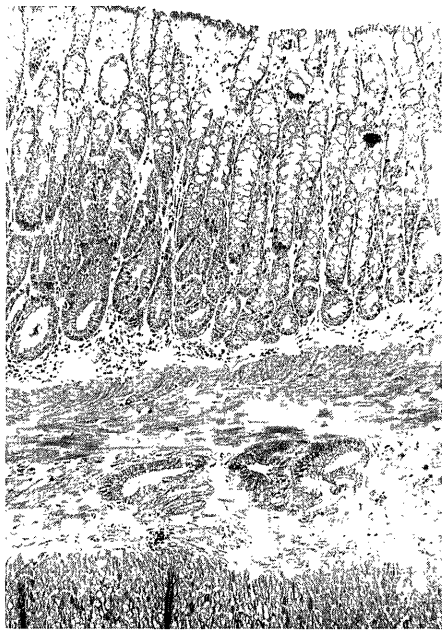

Figure 5. Epithelial gland under submucosa that was in no epithelial ulcer (X25).

Table 3. The relationship of existence of epithelial gland under submucosa between the depth of ulcer.

	Ul-0	Ul-2	Ul-3	Ul-4	total
-	8	9	4	2	23
1+	1	1	3	4	8
2+	1	1	8	3	13
3+	1	0	3	3	7
total	11	11	18	11	51

Table 4. Epithelial glands under submucosa and time course.

*()incidence(%)

	20w	30w	40w	50w	60w	total
-	1 (4.3)	1 (4.3)	1 (4.3)	5 (21.7)	15 (65.2)	23
1+	1 (12.5)	1 (12.5)	1(12.5)	5 (62.5)	0 (0.0)	8
2+	4 (30.8)	2 (15.4)	1 (7.7)	3 (23.1)	3 (23.1)	13
3+	0 (0.0)	2 (28.6)	3(42.9)	1 (14.3)	1 (14.3)	7
total	6	6	6	14	19	51

Table 5. Radiation injury score.

	20w	30w	40w	50w	60w
Macroscopic alterations of irradiated intestine	1.83±0.75	1.67±1.03	1.67±1.37	2.14±1.23	1.00±1.08
Mucosal ulceration	2.50±0.55	2.67±0.52	2.83±0.41	1.14±0.95	1.50±1.15
Epithelial atypia	1.83±0.75	2.17±0.41	2.00±1.26	1.29±1.07	1.33±1.08
Thickening of serosa	3.00±0.00	3.00±0.00	2.67±0.52	2.21±0.97	2.11±0.68
Vascular sclerosis	3.00±0.00	3.00±0.00	3.00±0.00	2.71±0.47	2.39±0.61
Fibrosis of intestinal wall	3.00±0.00	3.00±0.00	3.00±0.00	2.86±0.36	2.72±0.67
Iletis cystica profunda	1.83±0.98	2.17±1.33	1.83±1.47	1.36±1.08	0.61±1.04
Lymph congestion	0.83±0.41	1.00±0.00	0.50±0.55	0.64±0.50	0.67±0.49

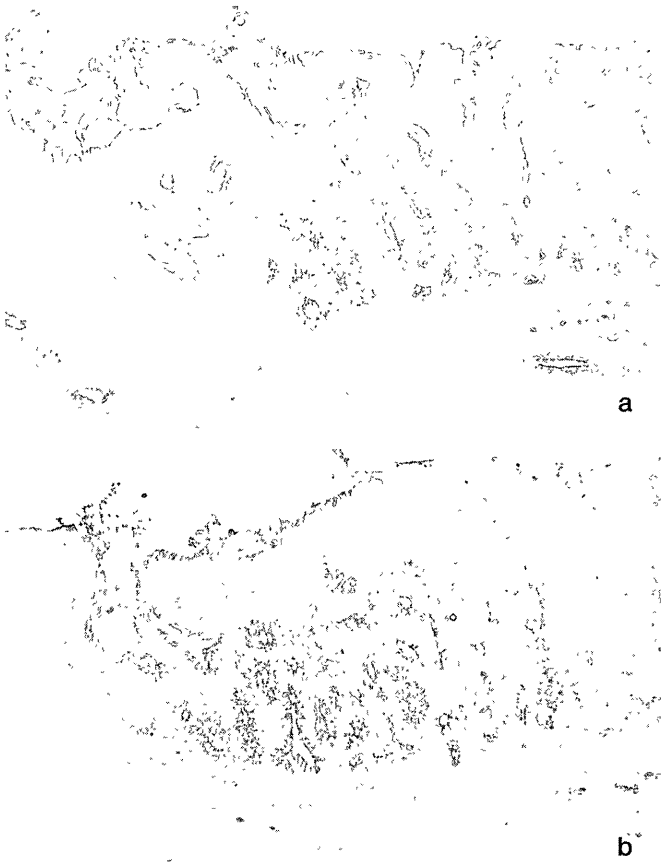

Figure 6. Urokinase expression in open ulcer at 20th week. Expression of uPA (a) and uPAR (b) was observed in epithelium beside open ulcer (X25).

Figure 7. Expression of uPA in epithelial cell under submucosal inclusion (X25).

was observed in 36 of 51 animals (70.6%). Submucosal fibrosis and thickening in irradiated field was observed in all cases of ulcer. Thickening and hyalinization of arterial wall was observed in submucosa. However, there was no finding of ischemic enterocolitis such as haemorrhage, necrosis, or

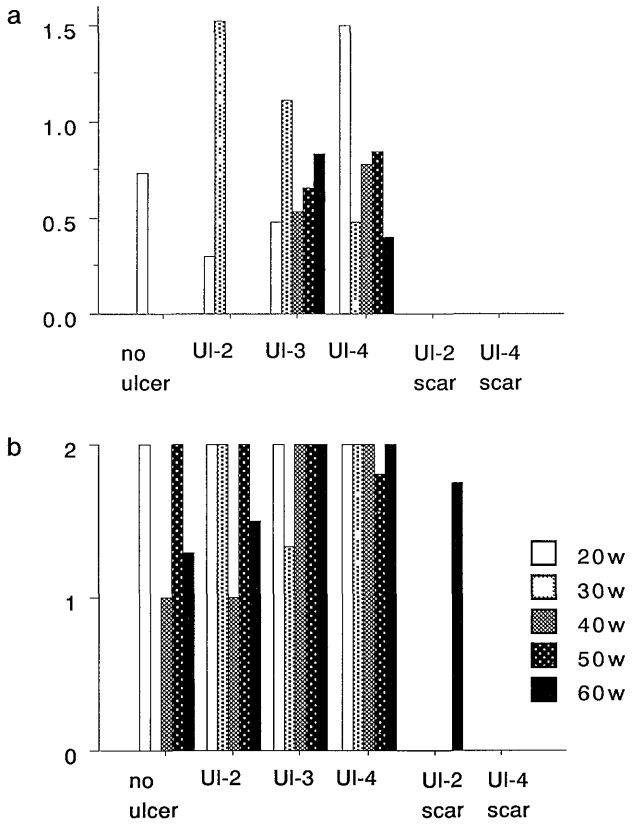

Figure 8. Expression of uPA and uPAR beside open ulcer.

pseudomembrane formation.

Total RIS showed sequential decrease; only epithelial factors of RIS decreased, while stromal factors of RIS remained at a high level after the 20th week (Table 5.).

(3) Immunohistological finding of urokinase

Expression of urokinase was higher in irradiated epithelium than in sham irradiated animals. While expression of uPA and uPAR was observed in epithelium and stroma, tPA was not expressed. uPA/ uPAR was expressed markedly in epithelium around the ulcers and epithelial glands under submucosa (fig. 6,7), and lower in fibrosis and venous wall except arterial wall. uPA in epithelial gland around the ulcer was expressed strongly, but less around the repaired ulcer. Expression of uPA showed sequential decrease. On the other hand, high expression of uPAR in epithelium was observed with the ulcers, healing ulcers and without ulcers, and this expression was high from 20th week to 60th week after irradiation (Fig.8.a-b). uPA expressions in repaired epithelium and epithelial glands under submucosa remained for a long time.

DISSCUSSION

Radiation induced enterocolitis in human manifests its symptoms after a latent period of several years. It is thought that our animal model can simulate human radiation induced enterocolitis. Acute radiation injury is thought to be caused by shedding of epithelial cells that is induced by direct DNA injury and/or indirect injury from free radical and secondary neutrophilic infiltration (12)(13)(14). It is still argued whether acute enterocolitis takes a prolonged course and becomes chronic, or repeats a healing and aggravating course. In human radiation induced enterocolitis, late radiation damage may occur without acute radiation injury.

Radiation induced enterocolitis is distinguishable from other forms of colitis by severe fibrous change in stroma and thickening or hyalinization of arterial wall (15). Radiation induced enterocolitis is never repaired within several weeks. While chronic radiation damage is thought to be a form of ischemic enterocolitis due to stromal fibrosis and severe vascular stenosis from vasculitis, there was no finding of ischemic colitis in any of the animals of our study.

The incidence of deep ulcers decreased after 40th week. Some ulcers were noted to have remained active as late as the 60th week, but their incidence was small. In our data, epithelial glands in submucosa without epithelial ulcer were observed in three of 51 animals (5.9%). The presence of epithelial gland in submucosa suggests uPA makes epithelial cells infiltrate to submucosal layer. Taking this into consideration, our model of radiation induced enterocolitis can be considered a model of repeated healing and aggravation rather than of continuous inflammation.

Urokinase is one of the proteinases discovered in conjunction with fibrinolytic system. Urokinase aggravates the ulcer by epithelial basal layer destruction. On the other hand, it is confirmed that urokinase has a role in repair of damaged epithelial cells in vitro. Subsequently, uPA-uPAR complex and uPAR itself promote migration of surrounding epithelial cells, and activated fibronectin acts to adhere migrated epithelial cells on epithelial basal layer (8). At same time, expression of uPA in fibroblast makes proliferation of fibrosis. In our study, the number of epithelial cells with high uPA expression was observed in case of increasing depth of ulcers. High expression of uPA is thought to deteriorate the ulcers rather than promote epithelial repair, if migration of epithelial cells is declined. Epithelial gland in submucosa that observed in high frequency is regarded as the basis of secondary malignancy (3)(4). High expression of uPA in epithelial cells may cause intestinal damage in radiation induced enterocolitis. We have to consider epithelial gland in submucosa as a center of recurrent inflammation and a basis of carcinogenesis.

In this study, ischaemic changes were not observed in irradiated colon despite sclerosing vasculitis. And expression of uPA was observed in the gland around ulcer, in the submucosal glands, and interstitial fibroblast. It is suggested that uPA played reciprocal roles in radiation induced enterocolitis: healing and aggravation of ulcer.

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