Table 4 CD177 expression in gastric carcinomas and its correlation with clinicopathological factors

	Case no.	CD177 Over-expression				*P* value‡
		Positive		Negative		
		Strong	Moderate	Weak	None	
Gastric adenocarcinomas	55	18	15	17	5	
Age						
Years (means ± SD)		55.3 ± 10.4	60.2 ± 8.13	59.8 ± 11.0	60.4 ± 13.0	0.5039
Histological classification						
Well/moderately-differentiated type*	21	6	9	4	2	0.1904
Poorly-differentiated/Signet-ring cell type**	34	12	6	13	3	
Depth of invasion†						
T1-3	27	5	10	10	2	0.2011
T4	26	11	5	7	3	
Lymph node metastasis						
N0	6	1	2	2	1	0.7869
N1-3	49	17	13	15	4	

* Lauren's intestinal type, ** Lauren's diffuse type, † Case number was reduced to fifty-three because the depth of invasion was not classified in two cases, ‡ ANOVA and Chi-square test were performed for age and other factors, respectively.

neoplasms. A number of rodent models of gastric cancer have been developed under various conditions, including *H. pylori* or *H. felis* infection, exposure to chemical carcinogens, and genetic modification [21,30]. Since *H. pylori* is known as a most closely-associated risk factor in man, animal models with infection of the bacterium, such as that utilizing Mongolian gerbils, are considered to be particularly important to mimic the background of human gastric carcinogenesis. On the other hand, there is a consensus that gastric cancer is a multifactorial disease [31]. Epidemiological studies and animal experiments have demonstrated that development of stomach cancer is also associated with many other factors including salt intake, alcohol drinking and cigarette, containing a wide variety of chemical carcinogen. In the present study, we attempted to mimic the gastric environment of human high-risk group exposed to combination of *H. pylori* infection, salt intake, and carcinogen.

As might be expected, there are both advantages and disadvantages of *Helicobacter*-infected mouse models. Instability of *cag* pathogenicity islands (PAI), a particularly important virulence factor of *H. pylori*, has been reported in the mouse model using SS1 strain [32]. Multiplicity of gastric tumors is difficult to examine in the gerbil model, because almost all of the stomach tumors in gerbils show invasive growth into the lamina propria or muscle layer. In the present study, our results demonstrated that *H. pylori* infection increased not only incidence but also multiplicity of gastric tumors in MNU-treated mice. Thus, the mouse model presented here has advantages in respect to investigate the multiplicity and tissue sampling for gene expression analysis.

In this study, we focused on the genes in which the expression was regulated only in *H. pylori*-infection and high-salt diet combined mice, which are expected to reflect the background of human high-risk group, to explore examples which might be associated with tumor progression. The two up-regulated genes selected, *Cd177* and *Reg3g* could be confirmed to exhibit significant over-expression by relative quantitative RT-PCR. Expression level of *Muc13* showed a tendency for increase with combination of *H. pylori* and salt, although this was not statistically significant. *Muc13* is a recently identified gene encoding transmembrane mucin that is expressed in the stomach to large intestine [33]. Shimamura et al. have reported that overexpression of *Muc13* is associated with differentiation towards the intestinal (differentiated) type of human gastric cancer [34]. In addition, the combined expression of

Table 5 Multivariate analysis of prognostic factos in patients with gastric cancer using Cox proportional hazard model

Factors	Hazard ratio	95% CI	*P* value
CD177 expression (negative)	2.07	1.063-4.021	0.0323
Age (year)	1.04	1.001-1.071	0.0439
Histological type (poorly-differentiated)	4.06	1.695-9.742	0.0017
Depth of invasion (high grade)	1.64	0.790-3.410	0.1838
Lymph node metastasis (positive)	3.40	0.773-14.92	0.1055

MUC13 with other metaplasia biomarkers is shown to be a prognostic indicator in several types of gastric cancer [35]. In the present study, all gastric tumors observed in MNU-treated mice were histologically of differentiated type. The REG protein family is also known to be associated with gastric cancer development and *Reg1α* and *Reg4* have been suggested as prognostic markers for advanced stomach cancers in man [36]. The present results indicate the possibility that *Reg3g* is also involved with progression of stomach tumor.

Immunohistochemical analysis of CD177 in advanced gastric cancer specimens showed expression to be significantly correlated with a good prognosis and survival rate after surgery. Importantly, multivariate analysis with clinicopathological factors as covariates further revealed high expression to be an independent prognostic factor for overall survival, as along with patient's age and histological classification. To our knowledge, the present study is the first to provide evidence that high expression of CD177 is associated with favorable prognosis in advanced gastric cancer.

CD177 is a member of the leukocyte antigen 6 (Ly-6) gene superfamily, encoding two neutrophil-associated proteins, NB1 and PRV-1 [37,38]. The NB1 glycoprotein is typically expressed on a subpopulation of neutrophils, located at plasma membranes and secondary granules. Recent studies have demonstrated that CD177 is overexpressed in neutrophils from 95% of patients with polycythemia vera and in half of patients with essential thrombocythemia [37]. Gonda et al. have reported a microarray analysis that *Cd177* expression in whole gastric tissue of *H. felis*-infected mice with mucosal dysplasia is reduced by folic acid supplementation [39]. Because they compared stage-matched groups to detect up- or down-regulated genes only by treatment of folic acid, it is unclear if *Cd177* expression is associated with gastritis or dysplasia. In our microarray results, there were no significant differences in expression of *Ela2*, which is a neutrophil-specific gene [40], and histological degrees of neutrophil infiltration were almost same among *H. pylori*-infected groups (data not shown). Therefore, the up-regulation of *Cd177* observed in this study was considered to be caused not by increased infiltration of neutrophils into the gastric mucosa but by a change of gene expression in tumor cells. NB1 is similar in structure to urokinase-type plasminogen activator receptor (uPAR), which is known to be associated with cell adhesion and migration [37]. Thus, there is a possibility that CD177 also acts as a regulator of adhesion and migration of neoplastic cells in gastric tumor. Further studies are needed to clarify the association between CD177 expression in gastric epithelial cells and tumor progression.

Conclusions

We demonstrated that the mouse model combined with *H. pylori* infection and high-salt diet is suitable for investigation of global gene expression associated with gastric tumor development and progression. Furthermore, our results suggest that CD177 expression might be associated with a favorable prognosis of gastric adenocarcinomas in man.

Abbreviations

FBS: Fetal bovine serum; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; H&E: Hematoxylin and eosin; *H. pylori*: *Helicobacter pylori*; Ly-6: Leukocyte antigen 6; MNU: *N*-methyl-*N*-nitrosourea; *Muc13*: Mucin 13; PAI: Pathogenicity islands; *Reg3g*: Regenerating islet-derived 3 gamma; RT-PCR: Reverse transcription-polymerase chain reaction; uPAR: Urokinase-type plasminogen activator receptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TTo and TTs designed the study under the supervision of AN, KO, TTa and MT. MY, HB, NS, ST, LS and AS participated in the animal handling and procedures. Clinical sample collection and suggestions were provided by SI and YY. Sample analysis and evaluation were performed by TTo, TTs and MY. All authors read and approved the final manuscript.

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Clinical Cancer Research

Cancer Therapy: Clinical
See commentary by Thompson and Gerner, p. 3509

Randomized Double-Blind Trial of Sulindac and Etodolac to Eradicate Aberrant Crypt Foci and to Prevent Sporadic Colorectal Polyps

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Abstract

Purpose: On the basis of the results of our preliminary trial suggesting that aberrant crypt foci (ACF) could be eradicated by short-term administration of sulindac, in the present study, we explored the feasibility of using ACF as surrogate markers for chemoprevention of colorectal cancer.

Experimental design: Randomly assigned to sulindac (300 mg daily), etodolac (400 mg daily), and placebo groups were 189 subjects without polyps or who had undergone polypectomy. Drugs were administered for 2 months. ACF in the rectal region were counted by magnifying endoscopy. Occurrence of polyps was evaluated at 12 months. A planned interim analysis was conducted.

Results: ACF number at 2 months was significantly suppressed in the sulindac group ($P = 0.0075$), but not in the etodolac group ($P = 0.73$). In the sulindac group, the numbers of adenomas plus hyperplastic polyps (total polyps) and adenomas at 12 months were significantly ($P = 0.02$) and marginally ($P = 0.064$) lower, respectively, in comparison with the placebo group; no such difference was observed in the etodolac group. In analysis of only polypectomized subjects, the numbers of total polyps and adenomas in the sulindac group were even more markedly lower, with P values of 0.014 and 0.034, respectively. A similar tendency was confirmed by analyses of the incidence of polyps at 12 months. Suppression rates of total polyps and adenomas in ACF responders to sulindac were significantly greater than in nonresponders. In all groups, compliance was more than 90% and no intolerable adverse effects were observed.

Conclusions: ACF may be useful as surrogate lesions for chemoprevention of colorectal cancer. *Clin Cancer Res; 17(11); 3803–11. ©2011 AACR.*

Introduction

Despite the recent introduction of various therapeutic modalities, colorectal cancer remains one of the most common causes of cancer deaths worldwide (1–3). Several chemopreventive modalities have been introduced in the past decade or so, and agents such as calcium (4), cyclooxygenase-2 (COX-2) inhibitors (5,6), aspirin (7–9), and sulindac, a nonsteroidal anti-inflammatory drug (NSAID; ref. 10), have been shown to inhibit the recurrence of colorectal polyps after polypectomy or the development of colorectal polyps. A major obstacle in the development of chemopreventive drugs is that they are administered for relatively long periods to cancer-free subjects; therefore, poor compliance and adverse effects frequently hamper trials.

We previously showed that aberrant crypt foci (ACF), tiny lesions that expressed the K-ras mutation and are identifiable only by magnifying endoscopy, correlated in number and size with the number of adenomas in patients with adenoma, and proposed these lesions to be precursors of colorectal adenoma and cancer (11–13). Subsequently, several investigators have confirmed our proposal of the ACF-adenoma-carcinoma sequence through demonstrating a close relationship between ACF and adenomas or cancers in terms of number, size, and pathologic features (14–19).

We then, though preliminarily, showed that ACF could be eradicated by short-term administration of sulindac (20,21) and proposed the possibility that discontinuous use of the drug may be just as effective as continuous use and may make the daunting task of chemoprevention more realistic. However, results of multicenter trials of

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Online registry: http://upload.umin.ac.jp; clinical trial no. C000000100.

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Translational Relevance

Trials for chemoprevention of cancer are generally daunting because drugs are administered for years to cancer-free subjects, resulting in low compliance and are sometimes associated with adverse effects. In the present randomized controlled study of chemoprevention of colorectal cancer, we successfully showed that a short-term (2 months) administration of a nonsteroidal anti-inflammatory drug (sulindac) to eradicate aberrant crypt foci, minute precursor lesions of polyps, was as effective as long-term administration of similar drugs in previously reported trials by using polyps as a surrogate marker for colorectal cancer. The results therefore suggest that a short-term and discontinuous administration of drugs is pertinent for chemoprevention because every cancer should be derived from precursor lesions (seeds), which could be readily eradicated by drugs.

chemoprevention by others have raised controversy over the ACF-adenoma-carcinoma sequence theory or the use of ACF as biomarkers for recurrent colorectal adenomas (22, 23). Results showed dissociation of ACF prevalence and adenoma history (22) or no significant modulation of ACF by celecoxib (23). However, accuracy as to the technical aspects of ACF detection in these studies might be questioned because the number of ACF detected was very low compared with that in other reports and inter-rater agreement rates were also low (11,14,15). Further, utilizing a COX-2 inhibitor might not have been appropriate in a trial to examine the effect on ACF because COX-2 was mostly negative in ACF and became positive in adenoma, although COX-1 was expressed in both ACF and adenoma (24).

In the present randomized controlled trial, to directly validate the usefulness of ACF as a surrogate marker for chemoprevention and to address the controversial issues described above, we explored the effect of short-term (2 months) administration of sulindac, an inhibitor of both COX-1 and COX-2, or etodolac, a selective COX-2 inhibitor, on ACF by employing magnifying endoscopy, which is a suitable method for detection of ACF as we reported previously (11,12). We also elucidated the relevancy of the effects of the drugs on ACF to that on polyp development 1 year after the start of the study.

Patients and Methods

Study design and subjects

This randomized, double-blinded, and placebo-controlled study was conducted between February 2002 and October 2007 at the 4th Department of Internal Medicine, Sapporo Medical University Hospital and its 4 affiliate hospitals. Actual recruitment was carried out from 2002 to 2006. The rather long-term recruitment period was because of the delay in approval by the institutional review board in 3 hospitals and the delay in dispensing the drug to 1 hospital. According to reduction rates in ACF number by sulindac administration in our preliminary trials (20, 21), we estimated that 360 subjects would generate 90% power for a difference in the ACF number among the groups. Because it was possible that the estimate of an adequate sample size to show significant efficacy of the investigational drug was too conservative, a planned interim analysis was carried out when half of the subjects had been enrolled.

Subjects were recruited from patients who had undergone colonoscopy for abdominal symptoms including discomfort, distension, and a feeling of tightness on defecation. Eligible criteria were (i) positive for ACF in the lower rectal region from the middle Houston valve to the dentate line, (ii) age from 20 to 75 years, (iii) no colorectal polyps or polyps had been resected by polypectomy, (iv) not pregnant, (v) no malignant disease, (vi) no active infection, (vii) no history of gastroduodenal ulcer, (viii) no use of NSAIDs or aspirin in the previous 2 months, (ix) no abnormal findings by laboratory tests [blood cell count, aspartate amino transferase, alanine aminotransferase (ALT), total protein, albumin, blood urea nitrogen, creatinine, total bilirubin, lactate dehydrogenase, creatine kinase, and electrolytes (Na, K, Cl)], and (x) no familial adenomatous polyposis.

Subjects were randomly assigned to 1 of the 3 treatment groups at a 1:1:1 ratio (sulindac, etodolac, or placebo) by an independent statistician in the study center. We administered each drug for only 2 months on the basis of our unpublished observation in the preliminary open trial, which showed that administration of sulindac brought about a significant reduction in ACF number in 6 of 7 patients with 2 months treatment, in 4 of 4 patients with 3 months of treatment, and in 5 of 5 with 5 months treatment, whereas in 6 patients with 1 month of treatment, the reduction rate was not significant. Therefore, in this study, the number of ACF was assessed after 2 months of drug administration (primary endpoint). The participants did not receive any further treatment, and assessment was made for the occurrence of polyps 1 year after initiation of the study (secondary endpoint).

There are reportedly 2 types of ACF, dysplastic, and non-dysplastic ACF, with the former type being suggested to be more likely a precursor of polyps than the latter (11, 17). However, in the present investigation, it was impossible to analyze these 2 types separately because the proportion of the dysplastic type among all ACF was too small for statistical analysis.

At the start of the study, we carried out a baseline colonoscopy on all patients to determine the presence of polyps in the entire colorectum and to count ACF in the rectal area. After 2 months of drug administration, we conducted only rectosigmoidoscopy on these patients to determine the number of ACF in the rectal area because the number of ACF in the rectal region correlates well with that in the total colorectum (11).

All patients provided written informed consent. The protocol and informed consent forms were approved

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by the institutional review board at each participating institution.

Endoscopy

Magnifying endoscopy (model EZW450, Fujinon-Toshiba ES System Co.) was used throughout the examination as previously reported (11, 12). The day before endoscopy, the patients consumed a low-residue diet, and were given orally 4 g magnesium sulfate and 5 mg of sodium picosulfate. On the day of endoscopy, 2,000 mL of polyethylene glycol (PEG) was given orally. When the feces were not sufficiently clear, they were given another 1,000–2,000 mL of PEG to ensure sufficient bowel cleansing.

A total of 5 endoscopists from Muroran Shinnittesu Hospital, Otaru Ekisaikai Hospital, Gorinbashi Hospital, and Sapporo Cancer Center Hospital, were engaged in the endoscopic examinations. They were all trained for at least 1 month at the 4th Department of Sapporo Medical University Hospital for detection of ACF. They were all blinded to the treatment arms. Each endoscopist carried out almost the same number of examinations in each arm.

At the baseline colonoscopy, the endoscope was inserted into the cecum, and the entire colorectum was carefully observed as the endoscope was pulled back. Insertion into the cecum was verified by videotape as described below. When the endoscope was pulled back to the rectum, the lower rectal region from the middle Houston valve to the dentate line was washed thoroughly with water, sprayed with 0.25% methylene blue, which was left to stand for 2 minutes, then washed again thoroughly with water. The number of ACF in the rectal region, which has been shown to correlate with that in entire colon (11), was counted by magnifying endoscope.

For the 2-month survey for ACF by rectosigmoidoscopy, we simply used a 110 mL glycerin enema to cleanse the region, and if the rectum was not sufficiently cleansed, the subject was orally administered 2,000–4,000 mL PEG.

One year after the initiation of the study, all patients underwent total colonoscopy to detect polyps in the entire colorectum. The same cleansing preparation was used as for the baseline colonoscopy. The same endoscopist carried out the baseline, 2-month, and 1-year endoscopic examinations for each subject. All procedures were recorded on videotape, and all ACF and polyps were photographed. The numbers of ACF and polyps were first counted by the operators during performance of the colonoscopy or rectosigmoidoscopy. To further ensure validity, another count was made through observation of the recorded videotapes by 3 expert endoscopists (M.M., Y.N., and Y.T.) from the Assessment Panel of Endoscopy.

Drug administration and monitoring for adverse effects

For blinding of subjects and trial staff, identical looking capsules were filled with either 150 mg of sulindac (Banyu, Tokyo, and Japan), 200 mg of etodolac (Wyeth Pharmaceutical Co. Ltd.), or 200 mg of lactose as the placebo. All subjects were also administered 15 mg of lansoplazole twice daily. The drug set for each subject was labeled by an identification code unrelated to the allocation to conceal the allocation from subjects and trial staff until the blind was opened.

Subjects were instructed to take 1 capsule after food in the morning and 1 capsule after food in the evening. Study patients visited the hospital every 2 to 4 weeks to be evaluated for subjective symptoms of any adverse events, including abdominal and cardiovascular symptoms and to receive a new supply of medication. Two weeks after initiation of the treatment, liver and renal function was evaluated. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) Version 2.0. Compliance was monitored by counting the capsules returned by patients every month at outpatient clinic. A Safety Monitoring Board reviewed the study semi-annually.

Statistical analysis

To maintain the overall type I error rate at 5%, Pocock's method (25) was applied for the interim analysis with a significance level of 0.0294. The number of ACF, the primary endpoint, was compared by the Mann–Whitney *U* test. For adjustment of multiplicity of 2 times pairwise comparison (placebo versus sulindac and placebo versus etodolac), the level of significance (0.0294) was modified by Bonferroni's method, that is, $0.0294/2 = 0.0147$. The number of polyps after 1 year was compared by Mann–Whitney *U* test. A comparison of the incidence of polyps after 1 year was made according to logistic regression analysis, and the level of significance was also set at $P = 0.0147$.

Results

Patients

A total of 304 subjects were screened for eligibility, and 115 subjects were excluded from the study for the reasons shown in Figure 1. The remaining 189 patients underwent randomization: 63 were assigned to the sulindac group, 64 to the etodolac group, and 62 to the placebo group (Fig. 1). Of these, 10 subjects withdrew their informed consent within 2 weeks: 5 after consulting with their family at home, 4 after deep reconsideration, and 1 after consultation with a supervisor at work. These subjects did not allow use of their data. Of the remaining 179, 4 were dropped from the study for taking NSAIDs for common cold, 1 for taking aspirin for headache, and 1 because of having orthopedic surgery during the study. The 177 patients (59, sulindac group; 60, etodolac group; 58, placebo group) underwent the 2-month endoscopy. The analysis was based on the intent-to-treat principle. Table 1 shows baseline characteristics of the subjects. No particular difference in each characteristic among the 3 groups was observed. In all groups, the number of polypectomized subjects was almost 5 times that of polyp-free subjects. No patient with hereditary nonpolyposis colorectal cancer (HNPCC) was included when we reviewed the subjects with regard to diagnostic criteria for HNPCC.

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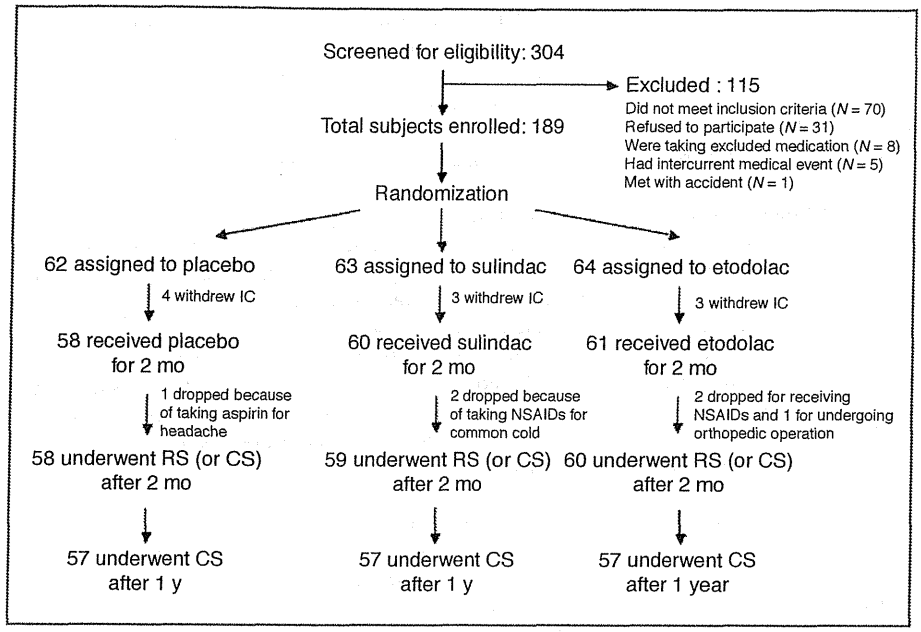

Figure 1. Trial profile. A total of 304 patients were screened for eligibility, and 115 were excluded. Enrolled were 189 patients who were randomly assigned to either the sulindac, etodolac, or placebo group. Drugs were administered for only 2 months, and the number of rectal ACF was assessed by rectosigmoidoscopy (RS). One year after the initiation of treatment, subjects underwent total colonoscopy (CS) to detect all polyps in the entire colorectum. The number of subjects is based on the intent-to-treat (ITT) population.

Validity of endoscopic assessment of ACF

The number of ACF in 26 patients randomly selected was evaluated through review of videotapes independently by 3 endoscopists from the Assessment Panel of Endoscopy (M. M., Y.N., and Y.T.) to assess the degree of concordance with the endoscopic findings. The mean counts of ACF were 8.5 ± 3.7 for M.M., 8.8 ± 4.6 for Y.N., and 8.6 ± 3.9 for Y.T. The inter-rater agreement rates within +/− 1 ACF between M.M. and Y.N., M.M. and Y.T., and Y.N. and Y.T. were 88.5%, 84.6%, and 92.3%, respectively. The Cronbach's alpha was 0.89, proving the validity of the endoscopic count of ACF.

Number of ACF before and after the 2-month treatment

The number of ACF before and after the 2-month treatment period in the 3 groups is shown in Table 2. In the polypectomized subjects, the ACF number after 2 months in the sulindac group was significantly lower than that in the placebo group ($P < 0.001$), whereas the number in the

Table 1. Baseline characteristics of subjects

	Placebo (n = 58)	Sulindac (n = 60)	Etodolac (n = 61)
Age, (mean ± SD), y	64.0 ± 9.9	65.8 ± 10.2	63.1 ± 9.7
Sex (M/F)	36/22	36/24	37/26
Colorectal cancer in parent no. (%)	8 (13.8)	8 (13.3)	10 (16.4)
Current smoker, no. (%)	11 (19.0)	12 (20.0)	11 (18.0)
History of diabetes, no. (%)[a]	5 (8.6)	6 (10.0)	5 (8.2)
History of hyperlipidemia, no. (%)[b]	14 (24.1)	15 (25.0)	16 (26.2)
History of hypertension, no. (%)[c]	18 (31.0)	20 (33.3)	18 (29.5)
Findings at baseline CS			
No. of polyps, median (interquartile range)	1.0 (0.5–2.0)	1.0 (0.0–2.0)	1.0 (0.0–2.0)
No. of adenomas, median (interquartile range)	1.0 (0.0–2.0)	1.0 (0.0–2.0)	1.0 (0.0–2.0)
Polypectomy/polyp-free subjects	48/10	50/10	50/11

[a]History of diabetes was defined as use of antidiabetic medication or participant report of clinically diagnosed diabetes.
[b]History of hyperlipidemia was defined as use of cholesterol-lowering medication or participant report of clinically diagnosed hyperlipidemina.
[c]History of hypertension was defined as use of antihypertensive medication or participant report of clinically diagnosed high blood pressure.

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Table 2. Comparison of ACF number among the sulindac, etodolac, and placebo groups before and after treatment

	Before[a]	After 2 mo	
Subjects with polypectomy			
Placebo			
Mean ± SD	7.77 ± 4.66	6.87 ± 6.03	
Median (interquartile range)	7.0 (5.0–10.0)	6.0 (3.0–8.5)	
Sulindac			$P < 0.001$[b]
Mean ± SD	7.70 ± 4.04	4.00 ± 2.95	
Median (interquartile range)	7.0 (4.5–10.0)	4.0 (1.0–6.0)	
Etodolac			
Mean ± SD	7.52 ± 4.01	6.28 ± 5.21	$P = 0.67$
Median (interquartile range)	7.0 (4.0–10.0)	5.0 (2.5–8.5)	
Polyp-free subjects			
Placebo			
Mean ± SD	4.00 ± 1.82	3.90 ± 2.72	
Median (interquartile range)	4.0 (2.0–6.0)	3.0 (1.0–7.0)	
Sulindac			$P = 0.38$
Mean ± SD	4.40 ±2.21	2.70 ±2.16	
Median (interquartile range)	4.0 (2.0–6.0)	3.0 (0–4.5)	
Etodolac			
Mean ± SD	4.73 ± 2.32	4.10 ± 2.60	$P = 0.54$
Median (interquartile range)	4.0 (0–5.5)	4.0 (2.0–9.0)	
Ali subjects			
Placebo			
Mean ± SD	7.12 ± 4.53	6.35 ± 5.69	
Median (interquartile range)	6.5 (4.0–9.0)	5.0 (2.3–8.0)	
Sulindac			$P = 0.0075$[b]
Mean ± SD	7.15 ± 3.98	3.77 ± 2.86	
Median (interquartile range)	6.5 (4.0–9.0)	4.0 (1.0–5.3)	
Etodolac			
Mean ± SD	7.01 ± 3.89	5.91 ± 4.93	$P = 0.73$
Median (interquartile range)	6.0 (4.0–9.0)	5.0 (2.0–7.5)	

[a] There were no significant differences before treatment among the 3 groups by the Kruskal-Wallis test.
[b] Significant *P* value by Mann–Whitney *U* test.

etodolac group was not significantly different from the placebo group ($P = 0.67$). Among polyp-free subjects, neither the sulindac nor etodolac group had a significant reduction in ACF compared with the placebo group. When polypectomized and polyp-free subjects were combined in the analysis, results were similar to those in the polypectomized subjects with a significant suppression of ACF ($P = 0.0075$) only in the sulindac group, reflecting the fact that polypectomized subjects were dominant in the subject population. Intraindividual comparison in ACF numbers before and after treatment (Supplementary Table S1) also showed a significant decrement in ACF in the sulindac group of polypectomized subjects ($P < 0.001$) as well as in all subjects in the sulindac group ($P < 0.001$). In the analysis of subjects receiving etodolac, ACF number tended to decline slightly after 2 months but without significance ($P = 0.09$). Thus, because the superiority of the test drug (sulindac) over the placebo was confirmed, termination of the study was recommended by the Data Monitoring Board. Incidentally, before the treatment, ACF number in subjects who had adenoma (subjects with polypectomy) was significantly higher than that in the subjects without adenoma (polyp-free subjects) in each group ($P < 0.001$), which was in good agreement with our previous finding (10).

Number and incidence of total polyps and adenomas 1 year after treatment

A total of 107 polyps were found after 1 year. Of these, 96 were adenomas and 11 were hyperplastic polyps (Supplementary Table S2). There was no significant difference in location and histology among the 3 groups. The average size in the sulindac group was slightly smaller than in the placebo group ($P = 0.16$ by Mann–Whitney *U* test).

In polypectomized subjects, the mean numbers of total polyps (adenoma plus hyperplastic polyp) and adenomas

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Table 3. Numbers of total polyps and adenomas 1 year after initiation of treatment

	Placebo	Sulindac	Etodolac
Subjects with polypectomy	$n = 48$	$n = 48$	$n = 47$
No. of total polyps			
Mean ± SD	0.92 ± 1.05	0.42 ± 0.68	0.73 ± 0.94
Median (range)	1 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.014$[a]	$P = 0.64$
No. of adenomas			
Mean ± SD	0.81 ±1.0	0.42 ± 0.71	0.68 ± 0.86
Median (range)	1 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.034$	$P = 0.61$
Polyp-free subjects	$n = 9$	$n = 9$	$n = 10$
No. of total polyps			
Mean ± SD	0.22 ± 0.44	0.22 ± 0.44	0.20 ± 0.42
Median (range)	0 (0–1)	0 (0–1)	0 (0–1)
P value		$P = 1.00$	$P = 0.94$
No. of adenomas			
Mean ± SD	0.11 ±0.33	0.22 ± 0.44	0.20 ± 0.67
Median (range)	0 (0–1)	0 (0–1)	0 (0–2)
P value		$P = 0.54$	$P = 0.93$
All subjects	$n = 57$	$n = 57$	$n = 57$
No. of total polyps			
Mean ± SD	0.81 ± 1.01	0.40 ± 0.70	0.68 ± 0.89
Median (range)	0 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.020$	$P = 0.61$
No. of adenomas			
Mean ± SD	0.70 ± 0.96	0.39 ± 0.68	0.60 ± 0.84
Median (range)	0 (0–4)	0 (0–2)	0 (0–3)
P value		$P = 0.064$	$P = 0.63$

[a]Significant *P* value by Mann–Whitney *U* test.

in the sulindac group were significantly ($P = 0.014$) and marginally ($P = 0.034$) lower, respectively, whereas those in the etodolac group were not lower with statistical significance ($P = 0.64$ for total polyps and $P = 0.61$ for adenomas) in comparison with the placebo group (Table 3). In polyp-free subjects, neither total polyp number nor adenoma number was lower in either the sulindac or etodolac group compared with the placebo group. In analyses of all subjects (polypectomized plus polyp-free subjects), the numbers of total polyps and adenomas were markedly ($P = 0.020$) and marginally ($P = 0.064$) lower in the sulindac group but not in the etodolac group. The incidences of total polyps and adenomas in polypectomized subjects were markedly ($P = 0.025$) and marginally ($P = 0.039$), respectively, lower in the sulindac group but not in the etodolac group in comparison with the placebo group (Table 4). In polyp-free subjects, there were no differences in incidence among the groups. When incidence was analyzed in all subjects, the incidence of total polyps was marginally lower ($P = 0.037$) and that of adenomas tended to be lower ($P = 0.08$) in the sulindac group but not in the etodolac group. Though statistically not significant because of the small number for analysis, there was a tendency ($P = 0.25$) for the incidence of multiple adenomas to decrease by treatment with sulindac; however, there were no apparent differences between the etodolac and placebo groups ($P = 0.81$).

Comparison of number and incidence of total polyps and adenomas between ACF responders and nonresponders to drugs

We selected out as responders those subjects whose ACF number became zero at 2 months or whose ACF reduction rate by 2 months was above the 90th percentile of the placebo group. We then compared polyp number and incidence at 12 months of the responders with those in the remaining subjects, which were designated as "nonresponders" (Supplementary Table S3). In the sulindac group, the numbers of total polyps and adenomas in responders were significantly lower than in nonresponders ($P = 0.017$ and $P = 0.023$, respectively). Moreover, the incidences of total polyps and adenomas in responders

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Table 4. Incidence of total polyps and adenomas 1 year after treatment

	Placebo	Sulindac	Etodolac
Subjects with polypectomy	$n = 48$	$n = 48$	$n = 47$
Incidence of total polyps	26/48 (54.2%)	15/48 (31.3%)	24/47 (46.8%)
Risk ratio (95% CI)		0.39 (0.17–0.89)	0.96 (0.43–2.15)
P value[a]		0.025	0.92
Incidence of adenomas	24/48 (50.0%)	14/48 (29.2%)	21/47 (44.7%)
Risk ratio (95% CI)		0.41 (0.18–0.96)	0.81 (0.36–1.81)
P value[a]		0.039	0.60
Polyp-free subjects	$n = 9$	$n = 9$	$n = 10$
Incidence of total polyps	2/9 (22.2%)	2/9 (22.2%)	2/10 (20.0%)
Risk ratio (95% CI)		1.00 (0.11–9.23)	0.88 (0.10–7.95)
P value[a]		1.00	0.91
Incidence of adenomas	1/9 (11.1%)	2/9 (22.2%)	2/10 (20.0%)
Risk ratio (95% CI)		2.29 (0.17–31.0)	2.00 (0.15–26.7)
P value[a]		0.53	0.60
All subjects	$n = 57$	$n = 57$	$n = 57$
Incidence of total polyps	28/57 (49.1%)	17/57 (29.3%)	26/57 (45.6%)
Risk ratio (95% CI)		0.44 (0.20–0.95)	0.87 (0.42–1.81)
P value[a]		0.037	0.71
Incidence of adenomas	25/57 (43.9%)	16/57 (28.1%)	23/57 (40.4%)
Risk ratio (95% CI)		0.50 (0.23–1.09)	0.87 (0.41–1.82)
P value[a]		0.08	0.70

[a]Logistic regression analysis was used to calculate *P* values.

were significantly lower than in nonresponders ($P = 0.011$ and $P = 0.022$, respectively). A similar tendency was observed but not with significance in the etodolac group.

Adverse effects and compliance

The incidence of adverse events, including symptoms such as abdominal pain, heartburn, diarrhea, and exanthema, and abnormal laboratory test results such as a transient elevation of ALT or creatinine was less than 4% (Table 5); all were grade 1. Differences were not significant among the 3 groups. No cardiovascular events, including myocardial infarction, angina, stroke, and transient ischemic attacks, were observed during the 2 months of treatment. Average compliance with medication was 92.7%: 93.9% in the placebo group, 91.7% in the sulindac group, and 92.5% in the etodolac group.

Discussion

For the present study, we selected 2 types of drugs, a NSAID (sulindac) and a COX-2 inhibitor (etodolac) because both were proved to be effective as chemopreventive agents for colon adenoma (10, 26–28). Drug dosages were selected according to information in previous reports (11, 29). Although one of the COX-2 inhibitors, high-dose celecoxib, was reported to increase the risk of cardiovascular events (30), we considered that our protocol, using etodolac in 1 arm, was safe because the cardiac adverse event related to etodolac was reportedly negligible at the dosage we used (31). We administered lansoprazole to all subjects, including those in the placebo group, to prevent any possible gastrointestinal damage caused by sulindac or etodolac.

In most previous chemopreventive trials for colon cancer, only the polypectomized subjects were enrolled (4–9). In the present trial, we recruited both polypectomized and polyp-free subjects in view of the possibility of differences between the 2 subject groups in sensitivity of ACF and polyps to drugs. However, to our disappointment, the number of polyp-free subjects enrolled was so small that we were, practically, not able to draw any meaningful conclusion from comparisons of these 2 groups. Incidentally, a possible reason for the high rate of polyps detected by the baseline colonoscopy was because patients who underwent the colonoscopic examination were those at high risk for colorectal polyps, such as those with fecal occult blood. Further, the relatively high proportion of polypectomized subjects compared with polyp-free subjects was probably because of their higher motivation to participate in the current trial. Nevertheless, the results of the 2-month treatment on ACF both in comparison analysis among groups (Table 2) and in the intragroup analysis (Supplementary Table S1) clearly indicated the effectiveness of sulindac in eradicating the lesions, particularly in polypectomized subjects. Thus, the primary endpoint of the present study was achieved. The failure of etodolac to eradicate ACF is probably explained by the fact that most ACF do not express COX-2 (20). Moreover, it is surmised

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Table 5. Incidence of adverse events and compliance

	Total $n = 179$	Placebo $n = 58$	Sulindac $n = 60$	Etodolac $n = 61$
Adverse events				
Abdominal pain	5 (3.0%)	2 (3.4%)	2 (3.3%)	1 (1.6%)
Heartburn	2 (1.1%)	1 (1.7%)	0 (0%)	1 (1.6%)
Diarrhea	2 (1.1%)	1 (1.7%)	1 (1.7%)	0 (0%)
Exanthema	1 (0.6%)	0 (0%)	1 (1.7%)	0 (0%)
Chest discomfort	1 (0.6%)	0 (0%)	0 (0%)	0 (0%)
Liver dysfunction[a]	2 (1.1%)	1 (1.7%)	0 (0%)	1 (1.6%)
Renal dysfunction[b]	2 (1.1%)	0 (0%)	1 (1.7%)	1 (1.6%)
Average compliance	92.7%	93.9%	91.7%	92.5%

[a]Liver dysfunction was defined as ALT level greater than the upper limit of normal.
[b]Renal dysfunction was defined as creatinine level greater than the upper limit of normal.

that in short-term treatment etodolac, which could not eradicate ACF, was ineffective in suppressing polyp development whereas sulindac was able to inhibit incidence of polyp 1 year after the initiation of treatment by eradicating ACF with short-term treatment.

Incidentally, intragroup analysis showed that in the placebo and etodolac groups, there was a slight tendency of a decrease in ACF number after 2 months although without statistical significance. At present, this cannot be explained but it can be speculated that subjects in these groups, as well as in the sulindac group, became very conscious of their dietary habits after enrollment in the study, which somehow influenced ACF occurrence. In this respect, the analysis among groups may be more reliable than intragroup analysis.

As to the relevance of histology of ACF (dysplastic and nondysplastic ACF) to their development into adenoma, no conclusive result was obtained in this study because 2 histologic types of ACF could not be analyzed separately because of the small proportion of dysplastic ACF in the total ACF population.

Explanation of the relevance of the effect of the drug on ACF to that on polyp development was another important task of the present study. Results showing in both analyses of the number and incidence of adenoma or total polyps either a significant or marked (marginal) reduction in the sulindac group strongly suggest not only the effectiveness of short-term treatment with sulindac in suppressing polyp occurrence but also the utility of ACF as precursor lesions for polyps although the possibility that the reduction in ACF was indirectly related to that of polyp occurrence cannot be completely denied. This notion was further supported by results of the analysis of responders versus nonresponders that showed significantly fewer polyps in the former than in the latter subjects in the sulindac group. Moreover, the average polyp size in the sulindac group was smaller than in the placebo group, although without statistical significance. Further, when the incidence of multiple adenomas was selectively analyzed, though statistically not significant because of the small number of patients, there was some tendency toward a decrease after sulindac treatment ($P = 0.25$). This also supports the notion that by suppressing ACF with sulindac, subsequent occurrence of adenoma may be reduced. In addition, the finding that the difference between the incidence of adenoma in polypectomized subjects in the sulindac group (29.2%) and that in the placebo group (50.0%) was almost the same as in previous studies in which aspirin or NSAIDs were administered over a long-term (7–9) suggests the possibility that 2 months of treatment may be as potent as 1 or 2 years in terms of adenoma prevention. Nonetheless, the suppressive effect of sulindac on adenoma in both the present study and in previous studies was not very substantial, that is, an up to 40% to 50% suppression rate. Thus, a future task is to develop a more effective drug, such as a specific inhibitor of GST-pi, which we showed in our previous reports to be quite potent in eradicating ACF (20, 32). Incidentally, the relatively high polyp recurrence rate after 1 year (43.9%), in agreement with that of a recent report (44.6%; ref. 6), may be because of advancements in endoscopic technology.

In conclusion, our results indicate that ACF may be more advantageous as surrogate lesions than adenomas for chemoprevention of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

A mechanism for abnormal angiogenesis in human radiation proctitis: analysis of expression profile for angiogenic factors

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Abstract

Background Radiation proctitis is an increasingly prevalent problem, with many patients being treated with radiotherapy for pelvic cancers. However, the mechanisms by which radiation proctitis develops in humans are not well understood. In this study, the expression profiles of angiogenic factors were analyzed to clarify their role in the etiology of radiation proctitis.

Methods Rectal biopsies were taken from 8 patients with radiation proctitis and 8 normal subjects. Protein lysates of the tissues were applied to an antibody array for angiogenesis-related factors. The mRNA level of each factor was evaluated by Taqman real-time PCR. Immunohistochemistry was performed using the labeled streptavidin biotin method.

Results Antibody array analysis revealed 2.12- to 7.31-fold higher expression levels of angiogenin, fibroblast growth factor 1 (FGF1), endoglin, matrix metalloproteinase (MMP)-8, urokinase-type plasminogen activator (uPA) and maspin in radiation proctitis tissues compared with normal rectal mucosa. The mRNA level of each factor in radiation proctitis tissue was significantly higher than in normal rectal mucosa, suggesting their transcriptional activation. Immunohistochemical staining showed strong expression of angiogenin and maspin in rectal epithelia, MMP-8 and uPA in infiltrating lymphocytes, FGF1 in fibroblasts and endoglin in endothelial cells. The expression of VEGF was not evident.

Conclusions Our results suggest that in radiation proctitis, MMP-8 and uPA cooperatively degrade the extracellular matrix and basement membrane to provide space for angiogenesis. Simultaneously, angiogenin and FGF1 promote endothelial cell proliferation, and endoglin induces vessel formation, culminating in angiogenesis. Inhibitors of angiogenic factors such as angiogenin and FGF1 may be effective for treating radiation proctitis.

Keywords Radiation proctitis · Angiogenesis · Angiogenic factor

Introduction

Radiation therapy is an important modality for cancer treatment as well as chemotherapy and surgery. It is a critical treatment method for intrapelvic malignancies such as cervical, prostatic, rectal and ovarian cancers. For cervical cancer, radiation therapy alone or a combination with chemotherapy (chemoradiation therapy), as well as surgery, is the first-line treatment for all clinical stages (stages I–IVa). For prostatic cancer, radiation therapy alone or a combination with endocrine therapy, as well as surgery, has been employed as first-line therapy for all cases with T1 to T4 without distant metastasis.

Although early radiation injury, which presents with local pain, mucus secretion and bloody stool, may occur

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during radiation treatment, most of these symptoms spontaneously disappear within a few months, and it rarely develops into a critical problem [1, 2]. In contrast, radiation proctitis is a form of late-radiation injury, occurring in 5–20% of cases treated with pelvic radiation, that typically emerges after 4 or more months post-irradiation and becomes the dose limiting toxicity for radiation therapy [2–4]. Radiation proctitis presents clinically with rectal bleeding, diarrhea, abdominal pain, fistulation and stenosis. The most common of these symptoms is rectal bleeding, which often impairs quality of life (QOL) and sometimes requires blood transfusion [5–7]. Radiation proctitis generally persists for life because there is no appropriate curative treatment.

Rectosigmoidal endoscopy in radiation proctitis shows proliferation of abnormal telangiectatic vessels and their bleeding, often accompanied by erosion and ulceration [8, 9]. Histopathologically, telangiectasia is seen with bleeding in the upper layer of the lamina propria and lymphocyte infiltration and fibrosis in the lamina propria [10, 11].

It has been reported that the main pathological findings in early radiation injury are acute inflammation and fibrosis caused by fibroblast proliferation, while in late radiation injury, abnormal angiogenesis is predominantly observed [10]. Liu et al. [12] investigated the expression of transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) at 1–3 months after irradiation of rat bowels and found that TGF-β was predominantly overexpressed at 1 and 2 months, causing fibrosis, and VEGF was expressed at 3 months, which induced angiogenesis. It was presumed that fibrosis and angiogenesis were induced by TGF-β and VEGF overexpression, respectively, in early radiation injury in animals.

Despite the significant clinical impact of late radiation injury, which is also characterized by abnormal angiogenesis, the role of angiogenic factors in this condition has not been investigated in animals or humans. Thus, the mechanism of onset of radiation proctitis remains to be elucidated. Should the mechanism be clarified, it may be possible to develop drugs to treat radiation proctitis with angiogenic factors as their target, and thus higher radiation doses could also be used, leading to improvement in cure rates.

In this study, the comprehensive expression profile of various angiogenic factors in tissues of patients with radiation proctitis was analyzed using an antibody array, and the mechanisms of onset were investigated. The analysis showed that the expression of VEGF, a major angiogenic factor expressed in animals with early radiation injury, was not evident, whereas angiogenin, fibroblast growth factor 1 (FGF1), maspin, matrix metalloproteinase-8 (MMP-8), endoglin and urokinase-type plasminogen activator (uPA) were overexpressed and supposed to accelerate abnormal angiogenesis in radiation proctitis.

Methods

Patients and samples

This study was approved by the Institutional Review Board at the University of Tokushima Graduate School. Written informed consent was obtained from all patients and healthy volunteers.

Eight patients with radiation proctitis (male/female, 6/2; average age, 67 ± 10.4 years) and 8 healthy volunteers (male/female, 5/3; average age, 65.3 ± 7.6 years) were enrolled in this study. Diagnosis of radiation proctitis was made according to the criteria of Cavcić et al. [13]. Briefly, the diagnosis requires a history of radiation therapy for an intrapelvic malignancy; characteristic colonoscopic findings such as erythema, edema, friability, ulcerations and telangiectasia; and histopathological exclusion of other diseases.

All 6 male patients with radiation proctitis received 70-Gy external irradiation for prostate cancer, and the 2 female patients received 50-Gy external irradiation and 20- or 27-Gy internal irradiation for uterine and vaginal cancers, respectively. All of the patients were referred to our department with the chief complaint of bloody stools. The mean period from the end of irradiation to the examination was 15.4 ± 10.2 months (range 7–38 months).

Biopsy samples were endoscopically taken from the telangiectatic lesions or non-telangiectatic lesions and snap frozen for antibody array analysis and Taqman real-time PCR, while the remaining samples were fixed in 10% formalin. The biopsy sites were treated by argon plasma coagulation. In the control group of healthy subjects, biopsy samples were taken at random from the rectal mucosa after total colonoscopy.

Antibody array

An antibody array (Proteome Profiler™ Array Human Angiogenesis Array, R&D Systems, Minneapolis, MN, USA), which could detect 55 angiogenic factors, was used according to the manufacturer's instructions. Briefly, fresh frozen samples that had been stored at −80°C were thawed, dissolved in lysis buffer [1% Triton-X-phosphate buffered saline (PBS)], and centrifuged to remove cellular debris. The samples were then incubated with biotin-labeled detection antibody for 1 h at room temperature. The sample–antibody mixtures were reacted with the membrane, on which capture antibodies for angiogenic factors were fixed, and incubated overnight at 4°C. The membrane was then washed and incubated with streptavidin-horseradish peroxidase (HRP) for 30 min. The membranes were developed using Chemiluminescent Detection Reagents (GE Healthcare, Buckinghamshire, UK) and visualized using a luminescent image analyzer (LAS3000-UVmini, Fujifilm, Tokyo, Japan).

Taqman real-time PCR

Total RNA was extracted using RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. The extracted total RNA was reverse transcribed into complementary DNA (cDNA) with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The concentration and amount of total RNA were determined by UV spectrophotometry. Probes and primers were from TaqMan gene expression assay reagents (Applied Biosystems). The following TaqMan gene expression assays were used: angiogenin (Hs00265741_s1, Applied Biosystems), maspin (Hs00184728_m1), MMP-8 (Hs01029057_m1), endoglin (Hs00164438_m1), uPA (Hs00170182_m1), FGF1 (Hs00265254_m1) and GAPDH (Hs99999905_m1) as an internal control. Quantitative PCR was performed on a 7500 Real Time PCR System (Applied Biosystems). PCR amplification conditions were 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The measured value was calculated by comparative threshold-cycle number (Ct) methods, and GAPDH gene amplification was used as an internal control. In order to determine the efficiency of each Taqman gene expression assay, standard curves were generated by serial dilution of cDNA, and quantitative evaluations of target and internal control gene levels were obtained by measuring Ct. Statistical significance was determined by the Mann–Whitney U test. Differences were considered significant at $p < 0.05$.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method with labeled streptavidin-biotin (LSAB, Dako, Kyoto, Japan), according to the manufacturer's instructions. Briefly, 3-μm-thick sections were cut from formalin-fixed paraffin-embedded tissues and mounted on slides. Sections were deparaffinized in xylene and hydrated in graded ethanol solutions (100, 95, 80, 70%) and PBS. The endogenous peroxidase was inactivated by incubation with 0.3% H_2O_2-MeOH. Subsequently, the slides were heated in 0.01 M citrate buffer in a water bath at 95°C (pH 6.0) for 15 min. Primary antibodies used were mouse anti-human CD31 monoclonal antibody (Dako), rabbit anti-human maspin polyclonal antibody (Sigma, St Louis, MO, USA), rabbit anti-human MMP-8 polyclonal antibody (Bioworld, Minneapolis, MN, USA), rabbit anti-human uPA polyclonal antibody (Bioworld), rabbit anti-human endoglin polyclonal antibody (Thermo Fisher Scientific, Fremont, CA, USA), goat anti-human angiogenin polyclonal antibody (R&D systems) and goat anti-human FGF1 polyclonal antibody (R&D systems). The sections were incubated with primary antibodies, washed with PBS and incubated with secondary biotinylated antibody from an LSAB+ peroxidase kit (Dako). Subsequently, the sections were incubated with HRP and visualized with DAB chromogen (3′,3-diaminobenzidine, Dako). Finally, the sections were counterstained with Mayer's hematoxylin.

Results

Endoscopic appearance and histological features of radiation proctitis

A representative endoscopic view of radiation proctitis from patients enrolled in this study is shown in Fig. 1a. Abnormally dilated blood vessels, namely telangiectasia, were observed in the lower rectum. These lesions existed in the superficial layer of the rectal mucosa, and the remaining intervening normal mucosa looked slightly pale. Histological examination of a biopsied specimen revealed obvious telangiectasia in the upper layer of the lamina propria and lymphocyte infiltration and fibrosis in the lamina propria (Fig. 1b). Immunohistochemical staining for CD-31, an endothelial cell marker, confirmed that telangiectatic lesions indeed existed in the upper layer of the lamina propria (Fig. 1c).

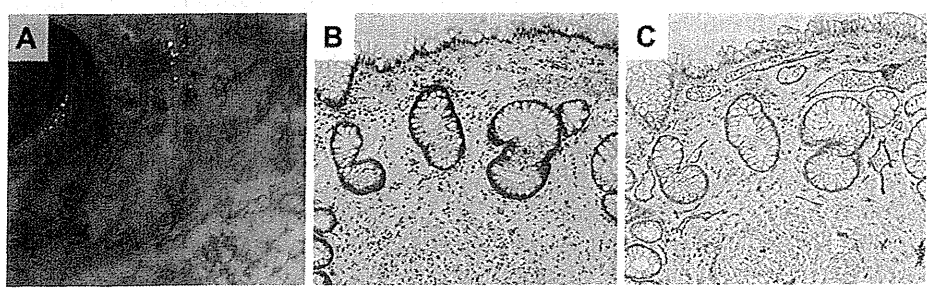
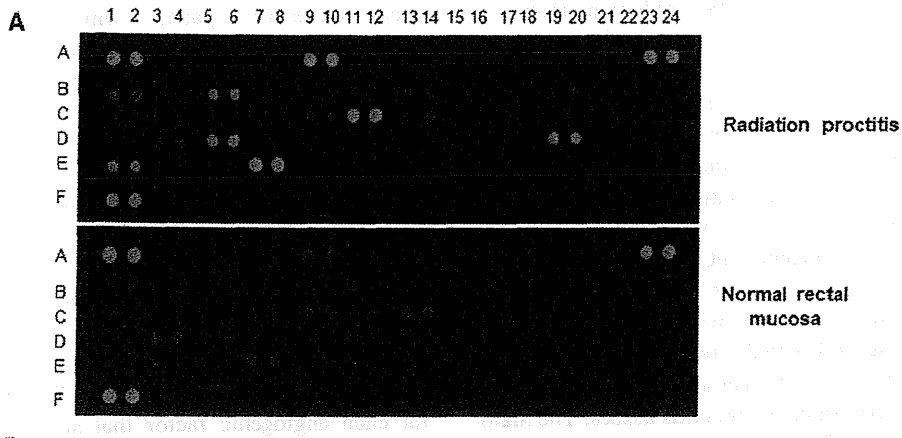


Fig. 1 Representative endoscopic and histological appearances of radiation proctitis. **a** Abnormally dilated blood vessels, a characteristic of radiation proctitis, were observed in rectal mucosa by endoscopy. **b** Telangiectasia and lymphocyte infiltration in the lamina propria were seen in H&E stained section (magnifications ×100). **c** Immunohistochemical staining for CD31, a marker for endothelial cells, confirmed that telangiectatic lesions existed mainly in the upper layer of the lamina propria



B

	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22	23,24
A	Positive Control		ActivinA	ADAMTS1	ANG	Ang1	Ang2	Angio-statin	AREG	Artemin		Positive Control
B	TF	CXCL16	DPPIV	EGF	EG-VEGF	Endoglin	Endostatin	ET1	FGF 1	FGF 2	FGF4	FGF7
C	GDNF	GM-CSF	HB-EGF	HGF	IGFBP1	IGFBP2	IGFBP3	IL1β	IL8	TGFβ1	Leptin	MCP1
D	MIP1α	MMP-8	MMP-9	NRG1β1	PTX3	PD-ECGF	PDGF-AA	PDGF-AB	Persephin	PF4	PLGF	Prolactin
E	Maspin	PAI1	PEDF	TIMP1	TIMP4	TSP1	TSP2	uPA	Vasohibin	VEGF	VEGF-C	
F	Positive Control											Negative Control

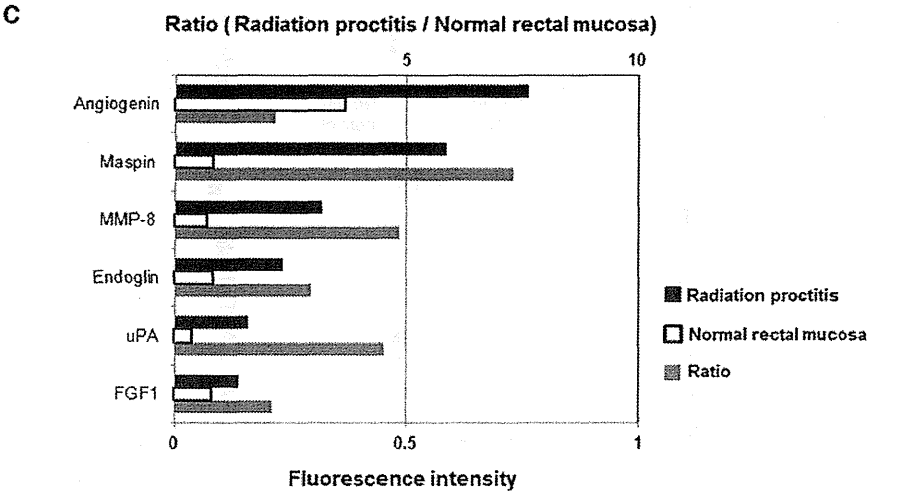


Fig. 2 The expression profile of angiogenic factors in radiation proctitis. **a** Expression of 55 angiogenic factors was detected by antibody array for radiation proctitis and normal rectal tissue. Three biopsy specimens from one patient were dissolved in the lysis buffer and applied to antibody analysis as described in "Methods." **b** The locations of antibodies for angiogenesis-related protein and positive and negative controls on the array are indicated on the chart as follows: *ADAMTS1* a disintegrin and metalloproteinase with thrombospondin motifs 1, *ANG* angiogenin, *Ang* angiopoietin, *ARGE* amphiregulin, *TF* coagulation factor III, *CXCL16* CXC chemokine ligand 16, *DPPIV* dipeptidyl-peptidase IV, *EGF* epidermal growth factor, *EG-VEGF* endocrine gland-derived vascular endothelial growth factor, *ET* endothelin, *GDNF* glial cell line-derived neurotrophic factor, *GM-CSF* granulocyte macrophage colony-stimulating factor, *HB-EGF* heparin-binding EGF-like growth factor, *HGF* hepatocyte growth factor, *IGFBP* insulin-like growth factor-binding protein, *TGF* transforming growth factor, *MCP* monocyte chemoattractant protein, *MIP* macrophage inhibitory protein, *MMP* matrix metallopeptidase, *NRG* neuregulin, *PTX* pentraxin, *PD-ECGF* platelet-derived endothelial cell growth factor, *PDGF* platelet-derived growth factor, *PF* platelet factor, *PLGF* placental growth factor, *PAI* plasminogen activator inhibitor, *PEDF* pigment epithelium-derived factor, *TIMP* tissue inhibitor of metalloproteinase, *TSP* thrombospondin, *uPA* urokinase-type plasminogen activator. **c** Expressions of angiogenin, FGF1, endoglin, maspin, MMP-8 and uPA were greater in radiation proctitis tissue than in normal rectal tissue

Identification of angiogenic factors in radiation proctitis by antibody array

To clarify the mechanism of abnormal angiogenesis, expression profiles of angiogenic factors in radiation proctitis were investigated in comparison with those in normal rectal tissue using the antibody array, which can detect 55 angiogenic factors. A representative result is shown in Fig. 2c. Angiogenin, FGF1, endoglin, maspin, MMP-8 and uPA were overexpressed by 2.12- to 7.31-fold in radiation proctitis as compared with normal rectal tissue. The same experiments repeated 3 times in 3 other patients also showed similar results in that all 6 angiogenic factors were overexpressed in radiation proctitis tissues. The major angiogenic factor, VEGF, was not overexpressed in radiation proctitis tissue compared with the normal rectal mucosa (0.91- to 1.32-fold). Antibody array analyses of rectal tissues from patients with active ulcerative colitis were also performed as a control applying the same method. Overexpression of angiostatin, angiopoietin-2, MMP-8, uPA, placental growth factor (PLGF) and interleukin-1β (IL-1β) were observed (Supplemental Fig. 1). Thus, the expression pattern of angiogenic factors in radiation proctitis was different from that in inflammatory bowel disease (ulcerative colitis).

mRNA levels of angiogenic factors in radiation proctitis tissue

The mRNA level was measured by Taqman real-time PCR for each angiogenic factor that showed protein overexpression in radiation proctitis tissue. The results showed that the mRNA levels of angiogenin, maspin, MMP-8, endoglin, uPA and FGF1 in the telangiectasia of patients

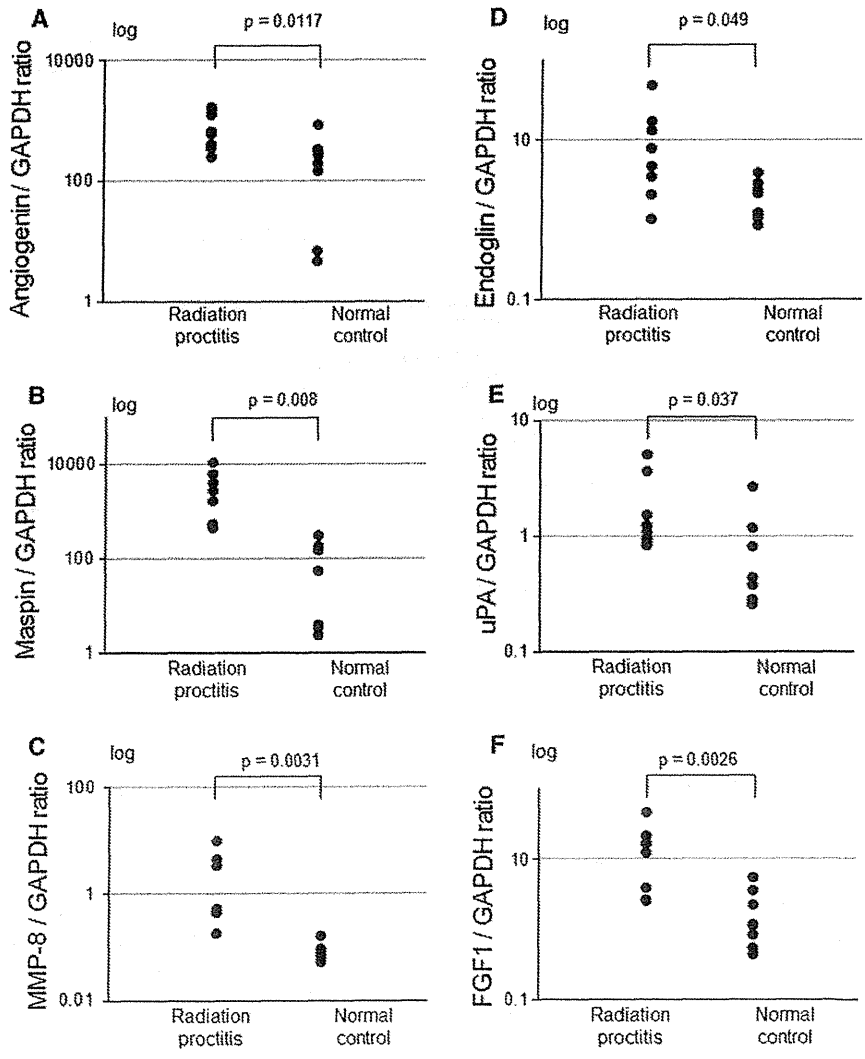


Fig. 3 Comparison of angiogenic factor mRNA levels between radiation proctitis and normal rectal mucosa. **a** Angiogenin; **b** Maspin; **c** MMP-8; **d** Endoglin; **e** uPA; **f** FGF1. The mRNA levels of all angiogenic factors in radiation proctitis tissue were significantly higher than those in normal rectal mucosa. Gene expression values were expressed as ratios between each angiogenic factor and an internal reference gene (GAPDH)

with radiation proctitis were significantly higher ($p < 0.05$) than those in normal rectal tissues (Fig. 3a–f, $p < 0.05$), indicating that these angiogenic factors were overexpressed by transcriptional activation.

The mRNA levels of angiogenic factors in the non-telangiectatic areas from patients with radiation proctitis were also analyzed (Supplemental Table 1). The mRNA levels of uPA, MMP-8 and FGF1 were significantly higher than in normal mucosa and were similar to those levels in telangiectatic areas. The mRNA values for angiogenin, maspin and endoglin were relatively higher than in normal mucosa, but without statistically significant differences.

The relationship between mRNA levels and the period after irradiation was also analyzed (Fig. 4). Interestingly, there were significant positive correlations between the mRNA levels of MMP-8, endoglin or FGF1 and the time after irradiation (from 7 to 38 months). However, no statistically significant correlations were observed between those values for angiogenin, maspin or uPA and the time after irradiation. Expression of the latter three factors remained at almost the same level and did not decline over time.

Immunohistochemical staining of angiogenic factors in radiation proctitis tissue

Since there are several kinds of stromal cells, including infiltrating lymphocytes, fibroblasts and endothelial cells, as well as epithelial cells in radiation proctitis tissues, immunohistochemical staining was performed to investigate the

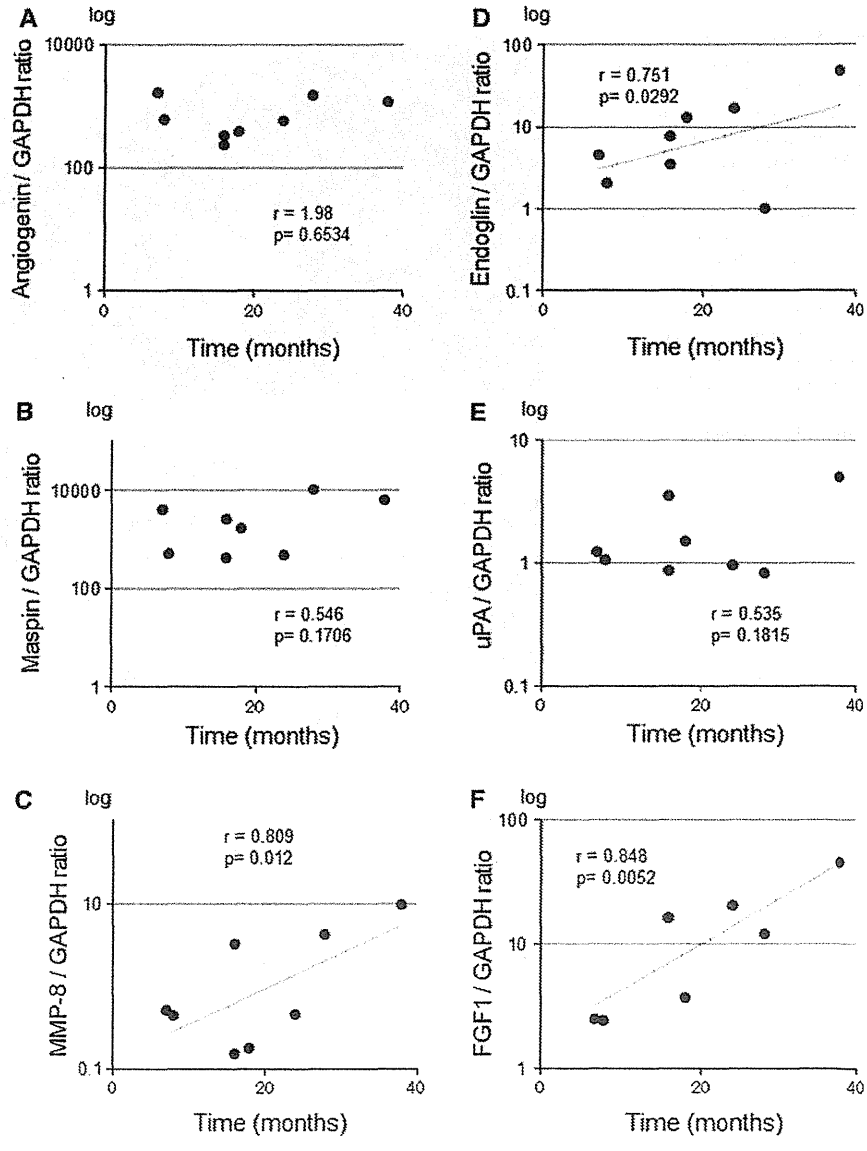


Fig. 4 The relationship between mRNA levels and the time after irradiation. **a** Angiogenin; **b** Maspin; **c** MMP-8; **d** Endoglin; **e** uPA; **f** FGF1. Significant correlations were observed between mRNA levels of MMP-8, endoglin or FGF1 and the time after irradiation (from 7 to 38 months). No significant correlations were observed between the mRNA levels of angiogenin, maspin or uPA and the time after irradiation

localization of each angiogenic factor. For each angiogenic factor, 5 different tissue specimens from radiation proctitis patients were stained; the representative staining patterns for each factor are shown in Fig. 5.

Angiogenin staining was sparsely positive in epithelial cells, particularly in the basal side of the epithelial cytoplasm (Fig. 5a). Although the reason for the sparse staining pattern was unclear, angiogenin was reported to be heterogeneously stained in prostatic neoplasms [14]. Maspin staining was strong in the cytoplasm of epithelial cells and weak in infiltrating lymphocytes (Fig. 5b). MMP-8 staining was evident in the cytoplasm of infiltrating lymphocytes and plasma cells. MMP-8 showed weak staining in epithelial cells (Fig. 5c). It was found that MMP-8 was positive in the CD20 (+) cells but not in the CD3 (+) cells by double immunofluorescence, indicating that MMP-8 was expressed in infiltrating B cells (Supplemental Fig. 2).

Staining for endoglin was intense in the cell membrane and cytoplasm of vascular endothelial cells of vessels (Fig. 5d). uPA staining was strong in infiltrating lymphocytes but was very faint in endothelial and epithelial cells (Fig. 5e). FGF1 staining was observed in fibroblasts, which often showed proliferation in radiation proctitis tissue, but not in epithelial cells or infiltrating lymphocytes (Fig. 5f).

Discussion

In this study, the protein expression profile of angiogenic factors was investigated for the first time in tissues of

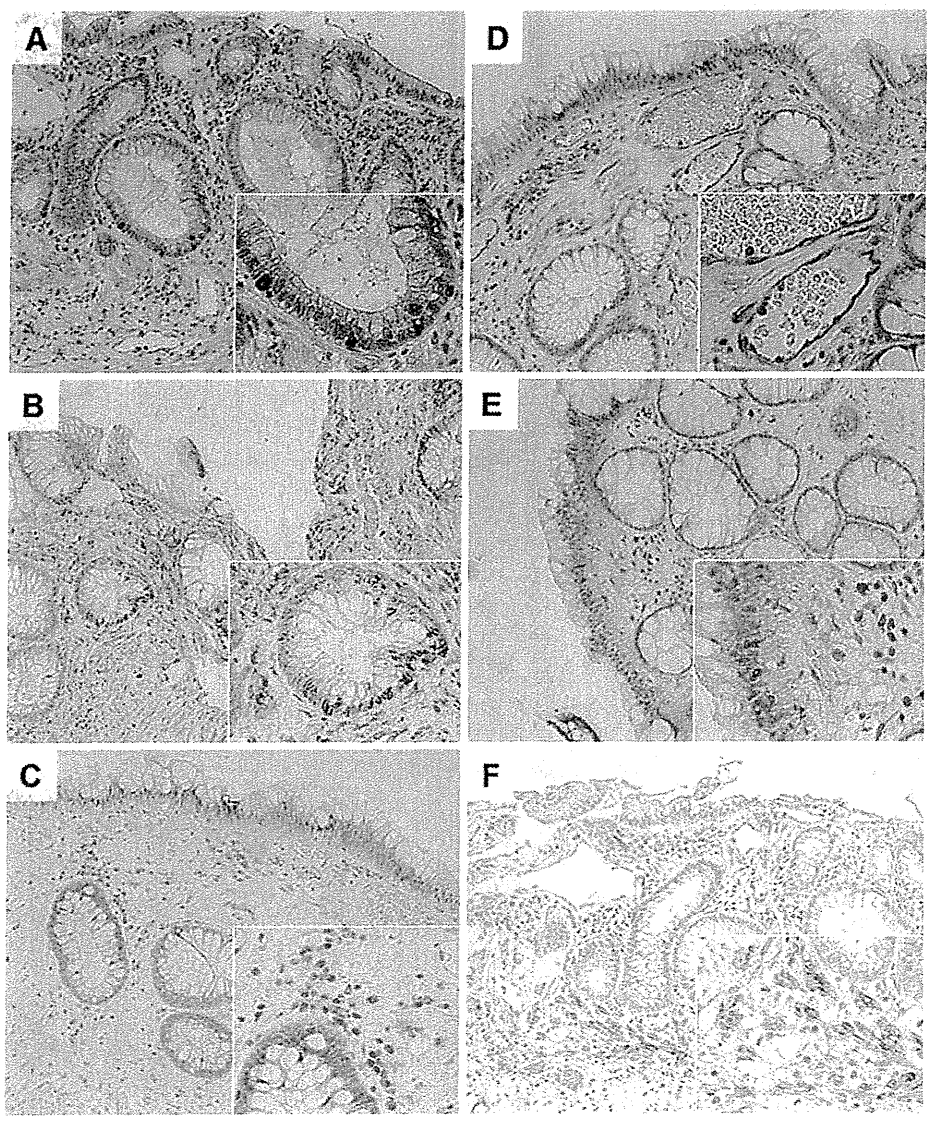


Fig. 5 Immunohistochemical staining for angiogenic factors in radiation proctitis. **a** Angiogenin showed sparse staining in epithelial cells. **b** Maspin showed strong staining in epithelial cells and weak staining in infiltrating lymphocytes. **c** MMP-8 was positive in infiltrating lymphocytes and epithelial cells. **d** Endoglin showed intense staining in vascular endothelial cells. **e** uPA showed strong staining in infiltrating lymphocytes but very faint staining in endothelial cells and epithelial cells. **f** FGF1 showed staining in fibroblasts. (magnification ×200, ×400)

humans with radiation proctitis, and it was demonstrated that angiogenin, FGF1, endoglin, uPA, MMP-8 and maspin were overexpressed in these lesions. It was also revealed that overexpression of these angiogenic factors was induced by transcriptional activation.

From the results of our experiments, the developmental mechanism of radiation proctitis in humans is envisioned as follows. First, MMP-8 and uPA, produced by infiltrating lymphocytes (B lymphocytes), cooperatively degrade the extracellular matrix (ECM) and basement membrane to provide space for angiogenesis. Simultaneously, angiogenin and FGF1, secreted from rectal epithelial cells and fibroblasts, stimulate proliferation of endothelial cells. Then, endoglin stimulates vessel formation by endothelial cells, leading to angiogenesis (Fig. 6). On the other hand, maspin is known to suppress migration and activation of endothelial cells. Therefore, maspin may inhibit angiogenesis in radiation proctitis. In the analysis of mRNA levels in relation to the time after irradiation, there were significant positive correlations between the mRNA levels of MMP-8, endoglin or FGF1 and the time after irradiation (7–38 months). However, the mRNA levels of angiogenin, maspin and uPA remained at almost the same level and did not decline over time. These results are consistent with the fact that radiation proctitis frequently develops within a few years and lasts for a long time of life.

It is well known that MMP induces the degradation of ECM and the basement membrane, whereas the tissue inhibitor of metalloproteinase (TIMP) inhibits MMP and promotes collagen formation in various organs. These two factors are physiologically well balanced in normal gastrointestinal mucosa as well as in other normal organs. However, in the patients with radiation proctitis, MMP was overexpressed, while TIMP was not, suggesting that ulceration and erosion are more likely to occur in these lesions. This is consistent with previous reports that radiation proctitis is often accompanied by rectal ulceration and erosion [15, 16].

Liu et al. [12] examined VEGF expression in rat rectal tissues at 1 to 3 months after 25 Gy (1 fraction) irradiation and found that it peaked at 3 months. Vujaskovic et al. [17] also reported that VEGF was overexpressed in rat lung tissues at 6 months after 28 Gy (1 fraction) irradiation. In the present study, at 7–38 months (15.4 ± 10.2 months) after irradiation the human radiation proctitis tissues did not express VEGF, but did express angiogenin and FGF1. We also confirmed by Taqman real-time PCR that there was no significant elevation of VEGF mRNA (data not shown). The difference between our data and those of Liu et al. or Vujaskovic et al. may be attributable to the difference in species studied or irradiation methodology.

It has been generally accepted that tissue hypoxemia induces angiogenic factors in late radiation injury. That is, irradiation impairs endothelial cells in the microvessels and causes hypoxemia. Subsequently, transcription factors such as hypoxia-inducible factor (HIF) are induced, and they then promote angiogenic factors such as VEGF, leading to angiogenesis [18, 19]. This postulation is based on in vitro experiments that showed that irradiation induced HIF in a cancer cell line and that HIF induced VEGF by binding to the promoter region of the VEGF gene [20–22]. In this study, however, there was no overexpression of VEGF. It is surmised that cancer cell lines inherently produce VEGF and therefore can easily increase VEGF expression

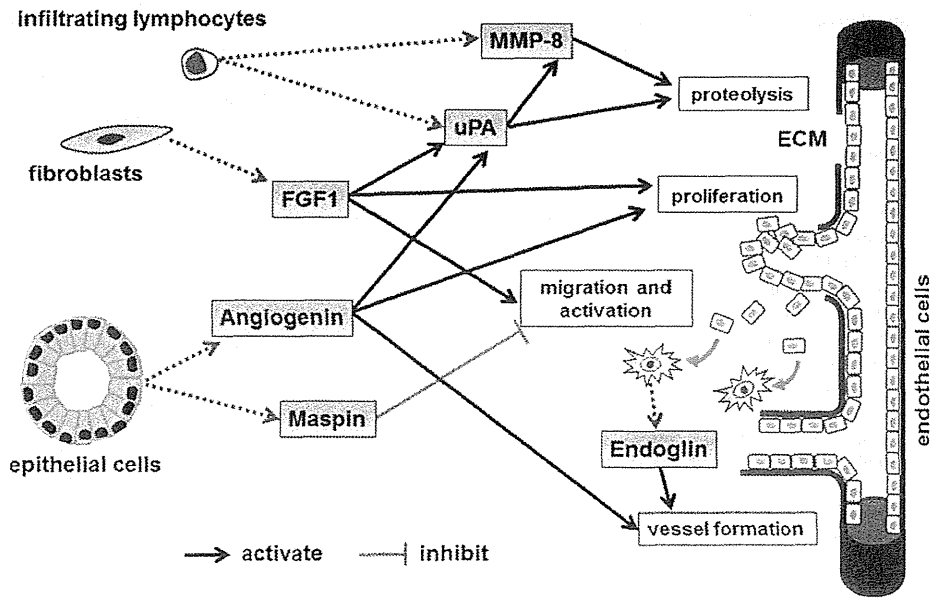


Fig. 6 The postulated mechanism of radiation proctitis development in humans. MMP-8 and uPA, produced by infiltrating lymphocytes, cooperatively degrade the extracellular matrix (*ECM*) and basement membrane to provide space for angiogenesis. Angiogenin and FGF1, secreted from rectal epithelial cells and fibroblasts, stimulate proliferation of endothelial cells. Endoglin stimulates vessel formation of endothelial cells, leading to angiogenesis