Jpn. J. Cancer Res. 88, 72–81, January 1997

Stromal Expression of MMP-9 and Urokinase Receptor Is Inversely Associated with Liver Metastasis and with Infiltrating Growth in Human Colorectal Cancer: A Novel Approach from Immune/Inflammatory Aspect

Satoshi Takeha,[1,2] Yoshihide Fujiyama,[2] Tadao Bamba,[2] Timo Sorsa,[3] Hiroshi Nagura[1] and Haruo Ohtani[1,4]

[1]*Department of Pathology, Tohoku University, School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-77,* [2]*Department of Internal Medicine, Shiga University Medical Science, Tsukinowa-cho, Otsu 520-21 and* [3]*Departments of Periodontology and Medical Chemistry, University of Helsinki, Helsinki, Finland*

MMP-9 (gelatinase B) and urokinase-type plasminogen activator receptor (u-PAR), which are involved in cancer cell invasion and metastasis, are reported to be predominantly expressed by immune/inflammatory cells in human colorectal cancers. To investigate their significance in cancer progression, we morphometrically analyzed the tissue expression of MMP-9 and u-PAR among different stages of colorectal cancer. The numbers of MMP-9- and u-PAR-positive cells along the invasive margin were significantly smaller in cases with liver metastasis than in cases without liver metastasis, and were also smaller in cases with an infiltrating margin than in cases with an expanding margin. Both variables were larger in colon cancer cases with conspicuous lymphocytic infiltration. These results indicated that the degree of tissue expression of MMP-9 and u-PAR by host cells is inversely associated with liver metastasis and an infiltrating growth pattern in human colorectal cancers. Essentially the same results were obtained for the number of macrophages distributed along the invasive margin. We also found that the expression pattern of MMP-9 was similar to that of MMP-8 (polymorphonuclear leukocyte collagenase). These data are consistent with clinicopathologic studies of host cells. Therefore, our data suggest a dual role of MMP-9 and u-PAR expression in colon cancer tissue; i.e., not only are these proteinases cancer-promoting factors, but also they are related to the host defensive mechanism when they are expressed by host cells.

Key words: MMP-9 — Urokinase receptor — Colorectal cancer — Host reaction — Liver metastasis

The host reaction to cancer cells has been recognized as one of the important factors that determine the biological behavior of cancers. Such reactions are usually most vigorous at the invasive margin, and various types of immune/inflammatory cells are involved, including T-lymphocytes, macrophages, and neutrophilic and eosinophilic granulocytes.[1] Of these, granulocytes are more abundantly distributed in human colorectal cancers than in the other cancers.[1] The immune/inflammatory infiltrate has been suggested to be a manifestation of the host reaction, being associated with a better prognosis in colorectal carcinomas as regards eosinophils[2,3] and lymphocytes.[4] The immune/inflammatory reaction and an expanding growth pattern at the invasive margin of rectal cancers are also reported to be favorable prognostic factors.[5] The liver is a representative site of hematogenous metastasis of colorectal cancer via the portal vein. Colorectal cancer with simultaneous liver metastasis is known to be characterized by less pronounced lymphocytic reaction[6] and a smaller number of macrophages[7] along the invasive margin. These results are consistent with the concept of an immune/inflammatory reaction taking place in cancer tissue.

Extracellular matrix (ECM) degradation has been shown to be prerequisite for cancer cell invasion and metastasis.[8] Many investigators have reported that certain proteinases including matrix metalloproteinases (MMPs) and matrix serine proteinases (MSPs) degrade the ECM, facilitating the invasion and metastasis of cancer cells.[9–11] MMP-9 (gelatinase B; 92 kDa type IV collagenase) belongs to the MMP family and degrades primarily type IV collagen and gelatin, and MMP-8 (polymorphonuclear leukocyte (PMN) collagenase; collagenase-2) is capable of degrading types I and III collagen.[12,13] Neoplastic as well as oncogene-transformed cells *in vitro* have been regarded as sources of gelatinases.[10,12] The plasma levels of MMP-9 were increased in patients with colon and breast cancers.[14]

Urokinase (urokinase-type plasminogen activator; u-PA), which is one of the MSPs, activates plasminogen into plasmin and initiates the plasmin cascade.[12,15,16] Urokinase is generated from pro-urokinase after binding to a specific receptor, urokinase receptor (u-PAR; CD87).[16] Plasmin can degrade the extracellular matrix

[4] To whom all correspondence should be addressed.

proteins directly and also indirectly by activating prometalloproteinases, thus contributing to cancer cell invasion.[16] Recent reports using human cancer tissues, however, have disclosed that several types of host cells including neutrophils, eosinophils and macrophages synthesize and/or store matrix-degrading enzymes.[17–19] u-PAR-protein has been reported to be localized in macrophages, granulocytes, and some cancer cells in human colon cancer.[20] These observations led to the concept that the inflammatory infiltrate facilitates cancer invasion and metastasis.[16–20] However, this concept seems to conflict with the above-mentioned view that immune/inflammatory cells are one of the manifestations of the host defense mechanisms against cancer invasion.[3,4,21] In fact, it is clear that the matrix-degrading proteinases are important in the regulation of inflammation and in tissue repair processes, with MMP-9 being involved at an early stage and MMP-2 at a later stage.[13]

To resolve this apparent discrepancy, we analyzed MMP-9 and u-PAR expression among different stages of colorectal cancer by means of morphometric analysis. We observed an inverse association between hematogenous metastasis and the expression of MMP-9 and u-PAR by host cells. We will discuss these unexpected results from the standpoint of the immune/inflammatory reaction against cancer invasion and propose that matrix-degrading factors can be regarded as manifestations of host defensive reactions.

MATERIALS AND METHODS

Tissues Formalin-fixed, paraffin-embedded tissues from 121 cases of colorectal cancer tissues were obtained from the files of the Department of Pathology, Tohoku University School of Medicine and Tohoku Rosai Hospital, Sendai, Japan. They included 41 cases in which cancer cells penetrated beyond the muscularis propria without metastasis (Dukes B stage), 42 cases with lymph node metastasis without hematogenous metastasis (Dukes C stage), and 38 cases with liver metastasis (Dukes D stage). Histologically normal-appearing mucosa attached to cancer tissue was also used as a control.

Our preliminary data showed that the host reaction is inconspicuous when the depth of invasion is in the muscularis propria rather than in the subserosa and submucosa. This may depend on the difference of stroma. We wished to standardize the host reaction, and so chose cases in which cancer cells penetrated the muscularis propria. Therefore, we did not include Dukes A cases and compared only Dukes B, C and D.

Antibodies The primary antibodies used (Table I)[22–24] were selected because of their applicability to paraffin-embedded sections. The absence of crossreactivity between anti-MMP-9 and anti-MMP-8 antibodies was confirmed by western blotting.[25]

Immunohistochemistry The indirect immunoperoxidase method was adopted for immunostaining. For MMP-9 and MMP-8 staining, the tissue sections (3 μm in thickness) were autoclaved after deparaffinization for 5 min in an aqueous phase for antigen retrieval as described previously (120°C, 2 kgf/cm^2 : 0.2MPa).[26] For u-PAR and CD68 staining, the tissue sections were pretreated with 0.1% trypsin for 25 min at 37°C. After these treatments, the tissue sections were incubated with the primary antibodies for 24 h at 4°C. The sections were then reacted with horseradish peroxidase-conjugated F(ab)$_2$ fragments of anti-mouse or anti-rabbit IgG (Amersham, Buckinghamshire, UK; diluted 1 : 100 in PBS containing 5% human serum) for 16 h at 4°C. The enzymatic reaction was developed for 10 min with 0.03% 3′-3 diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto) containing 0.006% hydrogen peroxide. The sections were counterstained with hematoxylin and mounted for light microscopy.

Our preliminary observation showed that the autoclave pretreatment for MMP-9 staining on paraffin-embedded sections gave essentially the same results as those with frozen sections (data not shown). The endogenous peroxidase activity was completely inactivated by incubation with 100% methanol containing 0.3% hydro-

Table I. List of the Primary Antibodies Used

Antibodies against	Source	Working dilution
MMP-9 (polyclonal)	Dr. L. Kjeldsen (Granulocyte Res. Lab., Copenhagen, Denmark)[22]	1 : 1000[a]
MMP-8 (polyclonal)	Dr. J. Michaelis (Christchurch Med. School, Christchurch, New Zealand)[23]	1 : 200[a]
u-PAR (monoclonal)	Dr. K. Danø (Finsen Lab., Copenhagen, Denmark)[24]	1 : 300[b]
CD68 (monoclonal)	DAKO	1 : 500[b]

a) With autoclave pretreatment.
b) With trypsin pretreatment.

gen peroxidase for 15 minutes after the incubation with the primary antibodies and by addition of 0.065% sodium azide in the DAB solutions. As the negative control, the primary antibodies were replaced by PBS or irrelevant rabbit antibodies. Pre-absorption of the anti-MMP-9 antibody with the purified antigen from polymorphonuclear leukocytes completely abolished the specific staining. For the identification of eosinophils, sections were stained with Chromotrope-R, which specifically stains eosinophil granules.

Morphometrical analysis of MMP-9 and CD68 The cells positive for MMP-9 and CD68 were quantified as reported previously[1] with some modifications. Briefly, three to five representative fields were selected along the invasive margin where average numbers of positive cells were observed after searching the whole field. In each field, immunoreactive cells were counted using an ocular grid (0.25×0.25 mm) at magnification $\times 400$. The counting was performed by two independent observers, and the level of disagreement between the two observers was always within a 15% range (data not shown). The average numbers of positive cells in each section were expressed per 0.0625 mm^2. We compared the result with those obtained using the other two counting methods that have been reported.[3,4] There were no significant differences among different cell counting methods (data not shown). The mean values of the three groups (Dukes B, C and D stages) were compared, and the statistical significance of differences was tested by the Tukey method.

Measurement of the u-PAR-positive area along the invasive margin Since u-PAR was not restricted to the cytoplasmic area and diffuse extracellular deposition was observed, we measured the area of u-PAR-positive cells as described below. A set of sampling lattices (composed of 225 lattices) was randomly superimposed on a microscopical image ($\times 40$) using the microscope with a tracing tube. The size of one lattice was 0.21×0.21 mm. First, we defined the line of the invasive margin, then the number of lattices in which u-PAR-positive cells were counted along the whole length of the invasive margin in each specimen. The degree of the u-PAR-positive area was defined as

$$D\ u\text{-}PAR = N/L \text{ (expressed as \%)}$$

where N is the total number of u-PAR-positive lattices, and L is the total number of the counted lattices along the invasive margin (L defines the total length of the invasive margin). This method mainly defines the continuity of the u-PAR-positive area along the invasive margin. Our preliminary study disclosed that the coefficient of error of this method was 5.7%. The mean values of the three groups (Dukes B, C and D stages) were compared, and the statistical significance of differences was tested by the Tukey method.

The number of u-PAR-positive cancer cells was counted per 1000 cancer cells in cases with immunoreactivity for u-PAR in cancer cells. The mean values of the three groups (Dukes B, C and D stages) were compared, and the statistical significance of differences was tested by the Tukey method.

Relationship to histopathological variables related to the survival of the patients: All cases were divided into expanding margin (99 cases; Fig. 1A) or infiltrating margin (22 cases; Fig. 1B) according to the pattern of cancer cell invasion, and into conspicuous (90 cases) or inconspicuous (31 cases) according to the degree of lymphocytic infiltration along the invasive margin, as described by Jass *et al.*[5] These two variables were related to the survival of the patients. The mean values of the

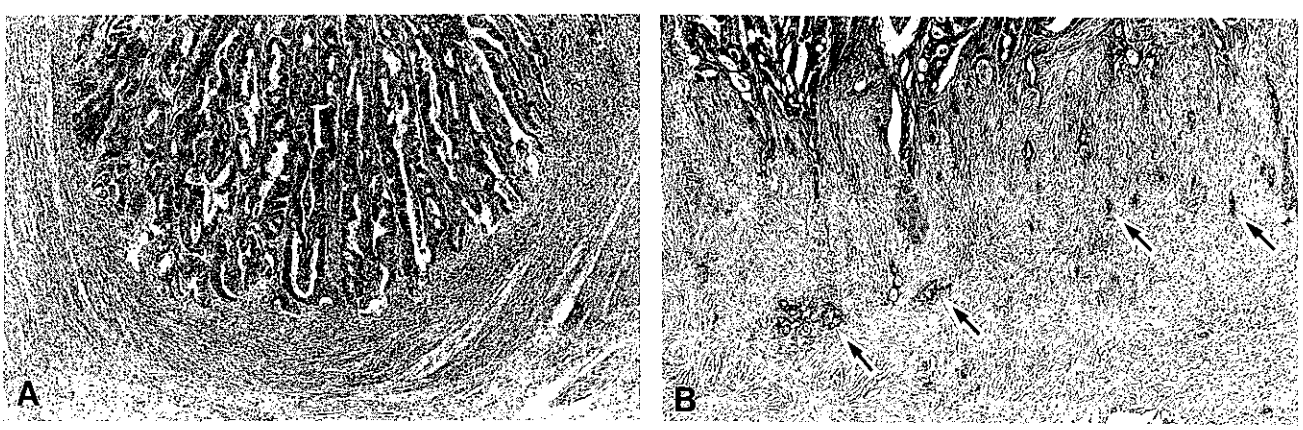

Fig. 1. Two invasive patterns of colon cancer cells; A, expanding margin and B, infiltrating margin. Arrows indicate cancer cells showing an infiltrating growth pattern. $\times 60$.

number of MMP-9-positive cells and the area of u-PAR-positive cells were compared between the two groups, and the statistical significance of differences was tested by use of the Mann-Whitney U test.

Discriminant analysis: To test the distinctness of each group, discriminant analysis was performed using a program "Seto/B" (Kyoritsu, Tokyo) for three variables (u-PAR, MMP-9 and CD 68) among Dukes B, C, and D groups, between expanding and infiltrating groups and between lymphocyte conspicuous and inconspicuous groups.

RESULTS

Immunohistochemistry for MMP-9 MMP-9-positive cells were distributed more abundantly along the invasive margin as a band-like pattern (Fig. 2). All positively stained cells were host cells and no reactivity was seen in cancer cells. Strongly positive cells were small, round cells with lobulated nuclei, and most of them failed to show chromotrope staining for eosinophils (data not shown). These cells were judged to be neutrophils. Other positively stained cells were oval or spindle-shaped, and were identified as macrophages on the basis of double immunohistochemical staining for CD68 and MMP-9. This result was in agreement with a previous result using the same antibody[19] (data not shown). MMP-9-positive cells were also abundant in the base of ulceration in cancer tissue.

Immunohistochemistry for MMP-8 Immunoreactivity for MMP-8 was mainly detected in granulocytes and in some macrophages. The distribution pattern was essentially the same as that of MMP-9 except that MMP-8-positive granulocytes were detected more frequently than MMP-9-positive cells in the neoplastic glands. The immunoreactive cells were abundantly distributed along the invasive margin. Cancer cells were negative (Fig. 3).

Immunohistochemistry for u-PAR u-PAR was positive in macrophages and granulocytes, which were distributed more densely along the invasive margin (Fig. 4), corroborating the previous reports.[20, 27] The immunoreactivity was also detected in the extracellular matrix around positively stained cells. Cancer cells were positive along the basolateral cell membrane and/or in the cytoplasm of several cells in a few cancer glands in 15 out of 121 cases. The immunoreactivity was also detected in the minute lumen as a secreted material in more than 20

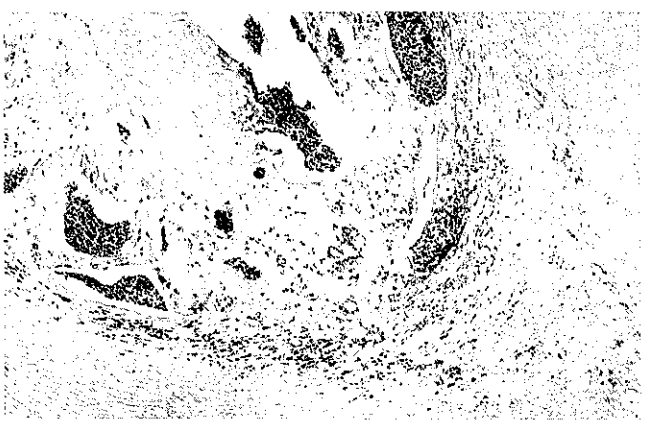

Fig. 3. MMP-8 expression in colon cancer tissue by immunohistochemistry. Note the similarity of this staining pattern to that of MMP-9 in Fig. 2. ×60.

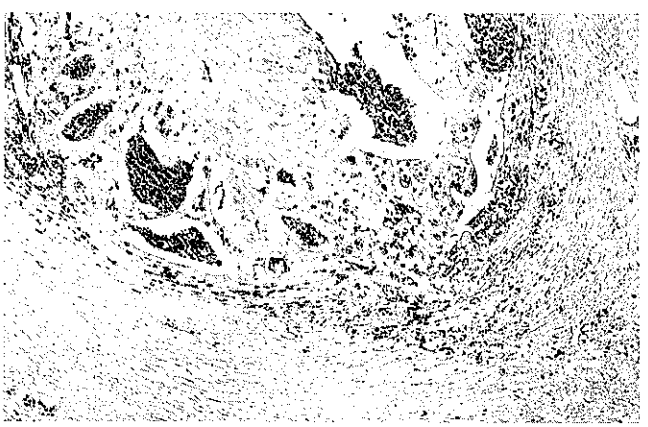

Fig. 2. MMP-9 expression in colon cancer tissue by immunohistochemistry (Dukes B case). MMP-9-positive cells are abundantly distributed along the invasive margin. Cancer cells are negative. ×60.

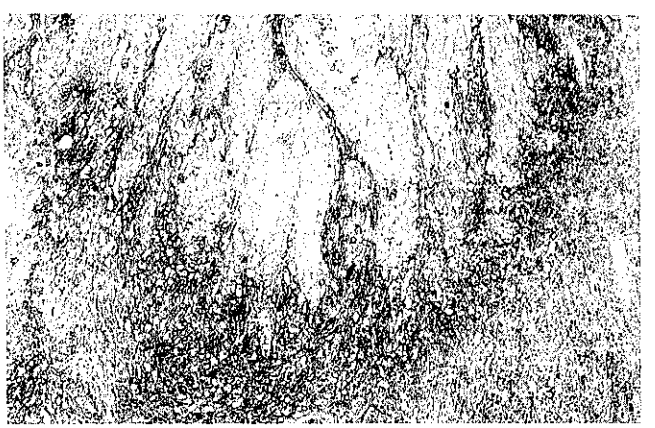

Fig. 4. u-PAR expression in colon cancer tissue by immunohistochemistry. u-PAR-positive stromal cells are distributed along the invasive margin (Dukes B case). ×60.

cases. We did not include this reactivity in the glandular lumen, because this staining pattern was not detected in frozen sections.[27]

Fig. 5. CD68-positive macrophages are distributed along the invasive margin (Dukes B cases). ×60.

CD68-positive macrophages were also densely distributed along the invasive margin (Fig. 5).

Morphometric analysis of MMP-9, u-PAR, and CD68 at different stages of colon cancer We compared the number of cells positive for MMP-9, the area of u-PAR-positive cells, and the number of macrophages detected by CD68 along the invasive margin among different stages of colon cancer. The average values of all three variables were significantly smaller in Dukes D cases (with simultaneous liver metastasis) than in Dukes B cases (cancer cells penetrating beyond the muscularis propria without metastasis) or than in Dukes C cases (with lymph node metastasis) (Fig. 6, A–C and Table II). There were no significant differences of the average values of any of the three variables between Dukes B and C cases.

Significant correlations were found between the values of u-PAR and MMP-9 (coefficient of correlation of 0.296; $P<0.001$), u-PAR and CD68 (0.371; $P<0.001$), and MMP-9 and CD68 (0.287; $P<0.001$).

To test the independence of each Dukes group, discriminant analysis was performed concerning all three

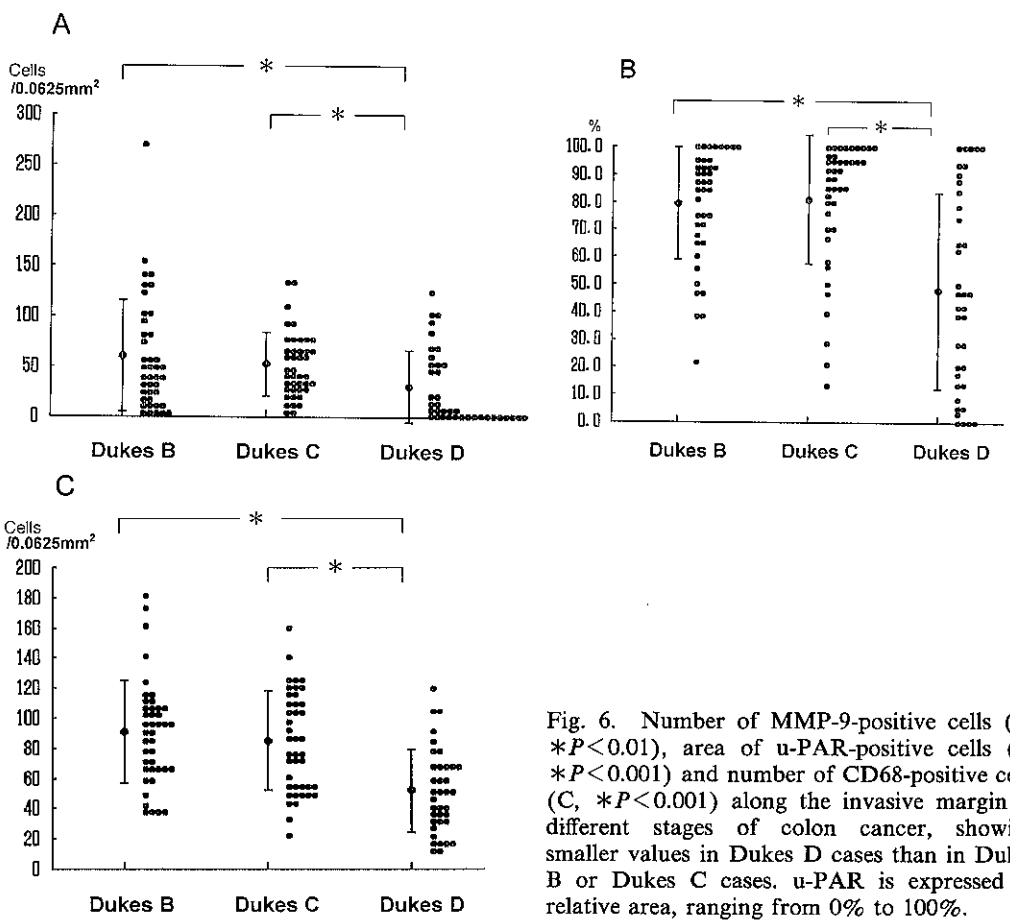

Fig. 6. Number of MMP-9-positive cells (A, $*P<0.01$), area of u-PAR-positive cells (B, $*P<0.001$) and number of CD68-positive cells (C, $*P<0.001$) along the invasive margin at different stages of colon cancer, showing smaller values in Dukes D cases than in Dukes B or Dukes C cases. u-PAR is expressed as relative area, ranging from 0% to 100%.

Table II. Results of Morphometric Analysis of Stromal Cells as Represented by Mean±1SD

	MMP-9	u-PAR	CD68
Stage			
Dukes B (41 cases)	60.7±55.0	79.5±20.5	90.9±34.2
Dukes C (43 cases)	52.9±31.6	81.0±23.4	84.8±32.9
Dukes D (38 cases)	30.2±35.9[b)]	47.8±35.7[a)]	51.9±27.4[a)]
Growth pattern			
Expanding (99 cases)	52.7±43.9	73.4±28.1	82.9±35.9
	$P<0.01$	$P<0.05$	$P<0.001$
Infiltrating (22 cases)	27.9±34.1	54.8±37.9	53.0±26.6
Lymphocytic infiltration along the invasive margin			
Conspicuous (90 cases)	55.8±45.1	74.9±28.0	86.1±34.9
	$P<0.001$	$P<0.01$	$P<0.001$
Inconspicuous (31 cases)	22.3±21.7	55.9±34.5	53.0±26.4

a) $P<0.001$ (smaller than in the other two groups), *b*) $P<0.01$ (smaller than in the other two groups). (MMP-9 and CD 68 are expressed as number per 0.0625 mm^2; u-PAR is expressed as % of area).

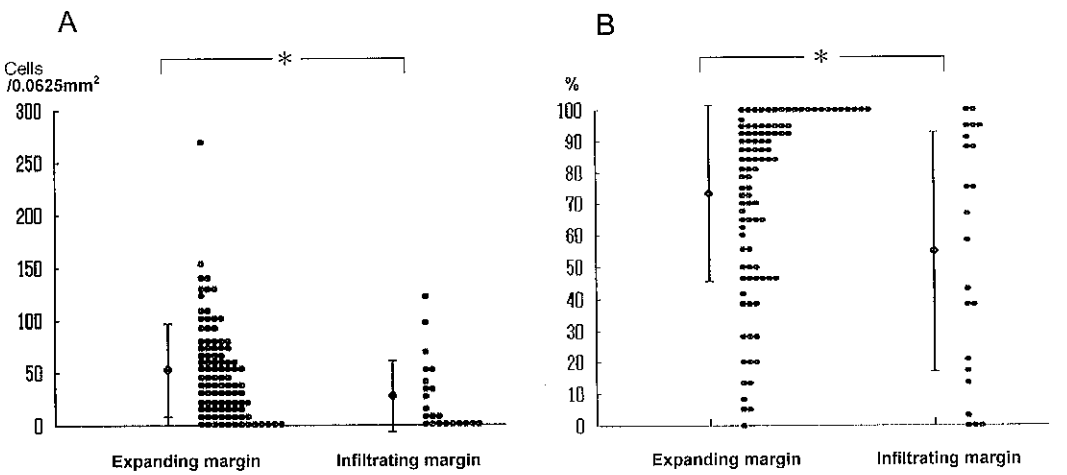

Fig. 7. Comparison of the number of MMP-9-positive cells (A, $*P<0.01$) and area of u-PAR-positive cells (B, $*P<0.05$) between infiltrating and expanding margins.

variables. Discrimination was confirmed between Dukes B and Dukes D, and between Dukes C and Dukes D cases. The validity of discrimination was 76.6% and 76.9%, respectively. CD68 ($P<0.001$) and u-PAR ($P<0.001$) contributed significantly to these two kinds of discrimination. Dukes B and C overlapped with each other.

Morphometric analysis between different patterns of invasive growth and between different degrees of lymphocytic infiltration The average values of all three variables (MMP-9, u-PAR and CD68) were smaller in cases with an infiltrating margin than in cases with an expanding margin (Fig. 7, A and B, Table II). The two groups were discriminated in discriminant analysis with a validity of discrimination of 72.9%. CD68 contributed significantly to this discrimination ($P<0.05$).

The average values of all three variables were smaller in cases with inconspicuous lymphocytic infiltration than in cases with conspicuous lymphocytic infiltration along the invasive margin (Table II). The discriminant analysis discriminated the two groups with a validity of discrimination of 77.1%. MMP-9 ($P<0.01$) and CD68 ($P<0.01$) contributed significantly to this discrimination.

u-PAR expression in cancer cells Among the 15 cases in which u-PAR was positive in cancer cells, the ratio of u-PAR-positive cancer cells was significantly larger in Dukes D cases (28.4±8.2) than in Dukes B cases (11.1±6.4) ($P<0.05$).

DISCUSSION

To our knowledge, this is the first paper to report a morphometric analysis of the tissue expression of MMP-9 and u-PAR in cancer tissue from the standpoint of the host inflammatory reaction. We have revealed that, in colon cancer, the host inflammatory cells expressing MMP-9 and u-PAR were less pronounced in cases with liver metastasis than in cases without liver metastasis and also they were smaller in cases with an infiltrating margin than in cases with an expanding margin. These results indicate the presence of an inverse association of matrix-degrading proteinases with liver metastasis and with infiltrating growth pattern. These unexpected results are difficult to explain in terms of the current concept of the molecular mechanisms involved in tumor invasion and metastasis. Therefore, we propose that the immune/inflammatory reaction, forming a part of the host's defensive mechanisms, is a significant source of matrix-degrading proteinases.

Cell identification in the present study was conducted essentially as previously reported.[19,20] MMP-9 was positive in macrophages and neutrophils. Of these, only macrophages are considered to produce MMP-9 *in situ*, as they are positive for MMP-9 mRNA[17] and they are immunolabeled in rough endoplasmic reticulum by immunoelectron microscopy (our unpublished data). Cancer cells were negative for MMP-9. This, however, does not necessarily exclude the possibility that cancer cells are one of the major sources of MMP-9-protein, since results may vary depending on the epitope(s) of each antibody used. u-PAR-protein was predominantly positive in granulocytes and partly in neutrophils. Macrophages in tumor tissues are designated as "tumor-associated macrophages (TAM)." TAMs are cytotoxic to cancer cells *in vitro* after stimulation.[21] Granulocytes (eosinophils) are known to have similar cytotoxic effects to cancer cells.[28] A recent study showed that IL-2-induced non-specific inflammatory responses by macrophages, neutrophils, and $\gamma\ \delta$ T-cells can lead to tumor rejection in athymic nude mouse.[29] Clinicopathological studies showed a positive correlation between eosinophil infiltration and a better prognosis of human colon cancer.[2,3] Therefore, the tissue degradation process by host cells can be regarded as a part of these host defensive mechanisms. In other words, insufficient host reaction can result in hematogenous metastasis and local invasive growth. Our current data showing that the expression of u-PAR and MMP-9 was more pronounced in cases with prominent lymphocytic infiltration along the invasive margin further supports this concept, since lymphocytic infiltration is one of the favorable prognostic factors of colorectal cancer.[5,6] Further support for this view is provided by the association between MMP-9 expression and the immune/inflammatory reactions; e.g., cell-to-cell contact between activated T-lymphocytes and monocytes induces the expression of MMP-9 by monocytes as an important step of inflammation and ECM destruction.[30] Thus, it is not surprising that complex expression patterns of MMP-9 are observed in tissue extracts of colon cancer at different stages.[31] u-PAR-expression has also been reported to be associated with immune/inflammatory reactions; e.g., a) the expression of u-PAR on the surface of monocytes was up-regulated by cytokine stimulation,[32] and b) u-PAR may facilitate migration and extravasation of T-lymphocytes.[33]

Direct visualization of cell-to-cell contact between intercellular adhesion molecule 1^+ (ICAM-1^+) macrophages and lymphocytes further strengthens the concept of immunologic activation of macrophages distributed along the invasive margin.[34] This stands in contrast to macrophages within colon cancer tissue, which exhibit a phenotype of immunologic unresponsiveness.[35] As regards the entrance of these immune/inflammatory cells to the locale, we have observed the occurrence of venules expressing E- and P-selectins along the host side of the invasive margin of colon cancer.[7]

Another viewpoint is to look at the turnover (production and degradation) of the extracellular matrix in cancer tissue. MMP-9 also degrades fibrillar types I and III collagen, which are partly degraded by other proteinases.[12,13] Cancer invasion usually induces an overproduction of collagen (desmoplastic reaction) in the stroma.[36] MMP-8, which efficiently degrades fibrillar type I collagen,[13] is also abundantly expressed, predominantly by granulocytes and partly by macrophages, in a pattern similar to that of MMP-9 in our cases. A recent study showed that MMP-8 mRNA and protein can be found in non-granulocytic cells.[37] Therefore, it is also conceivable that MMP-9, MMP-8 and MSPs act cooperatively as turnover enzymes of the extracellular matrix, as proposed in gastrointestinal[38] and pancreatic[39] cancers. u-PAR can also take part in this process by activating pro-metalloproteinases.

A recent study on the ability of colon cancer cells to induce the production of metalloproteinases in monocyte-lineage cells (THP-1) *in vitro*[40] indicated that metastatic colon cancer cells induced higher levels of both MMP-2 and MMP-9 in THP-1 cells than non-metastatic cells did. The differences in the results may be explained by the difference of methodology adopted; we analyzed the *in vivo* distribution of immunoreactive cells.

Plasmin-mediated proteolysis is also important in the wound healing process, since wound healing is retarded in plasminogen knock-out mice.[41] This study suggested that fibrin degradation in ulcer bases is the first important step in wound healing. This concept may be applicable to the present study, because the host reaction to cancer invasion shares many similarities with wound healing.

First, the aggregation of macrophages, neutrophils and lymphocytes observed along the invasive margin of colon cancer is common to the acute inflammatory changes in wound healing. Second, fibrosis, the next important step of wound healing, is also observed as a desmoplastic reaction in the stroma and also in the so-called reactive fibrosis zone of colon cancer.[38] These considerations indicate the importance of further comparative studies between the host reaction in cancer tissue and the inflammation and/or wound healing process.

We dealt only with intracellular latent MMP-9. Secreted MMP-9 is converted into the active form by the cleavage of its prodomain in the extracellular matrix. A microdissection study reported that the active form of MMP-9 was detected in colon cancer tissue which contained necrosis and/or a marked degree of inflammation,[42] which is consistent with our data on MMP-9 localization. Tissue inhibitors of matrix metalloproteinase (TIMPs) are also important, competing with MMPs to downregulate ECM degradation.[11, 12]

Regarding the possible function of MMP-2 (gelatinase A), we have recently proposed that MMP-2 produced by activated fibroblasts is involved in matrix turnover in the desmoplastic reaction occurring in cancer stroma and in the reactive fibrosis zone of cancer tissue.[38] Considering the differences of cell sources of MMP-2 and MMP-9, we speculate that, in the tissue reaction, MMP-9 functions in an earlier stage and MMP-2 in a later stage, because MMP-9 originates from macrophages and neutrophils while MMP-2 is secreted predominantly from fibroblasts.

On the basis of the above considerations, we propose that granulocytes and macrophages, together with T-lymphocytes, function defensively for the host against the hematogenous metastasis and the local invasive growth of human colorectal cancer, and that the matrix degradation proteinases/factors are derived from these host cells to a significant extent. Therefore, matrix degradation processes in malignant tumors have two distinct aspects; i.e., matrix degradation induced by cancer cells as a tumor-promoting factor and that induced by host immune/inflammatory cells as a defensive mechanism. Further clinicopathological and basic studies are required to clarify the implied duality of matrix degradation processes in cancer tissue.

ACKNOWLEDGMENTS

We are grateful to Dr. Tohru Takahashi and Dr. Hiroshi Yaegashi, Department of Pathology, Research Institute for Developmental Biology and Cancer, Tohoku University, for their advice on morphometrical analysis, to Dr. Yukimasa Suzuki, Department of Pathology, Tohoku University School of Medicine for helping us to count MMP-9-positive cells, to Dr. Ikuro Sato, Department of Pathology, Miyagi Prefectural Cancer Center for the statistical analysis and to Dr. Keld Danø, Finsen Laboratory, Copenhagen, Denmark for providing anti-uPAR antibody. This study was partly supported by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan.

(Received June 3, 1996/Accepted October 30, 1996)

REFERENCES

1) van Ravenswaay Claasen, H. H., Kluin, P. M. and Fleuren, G. J. Tumor infiltrating cells in human cancer. *Lab. Invest.*, **67**, 166–174 (1992).
2) Pretlow, T. P., Keith, E. F., Cryar, A. K., Bartolucci, A. A., Pretlow II, T. G., Kimball, P. M. and Boohaker, E. A. Eosinophil infiltration of human colonic carcinoma as a prognostic indicator. *Cancer Res.*, **43**, 2997–3000 (1983).
3) Fisher, E. R., Paik, S. M., Rockette, H., Jones, J., Caplan, R. and Fisher, B. Prognostic significance of eosinophils and mast cells in rectal cancer. *Hum. Pathol.*, **20**, 159–163 (1989).
4) Harrison, J. C., Dean, P. J., El-Zeky, F. and Zwaag, R. V. Impact of the Crohn's-like lymphoid reaction on staging of right-sided colon cancer. *Hum. Pathol.*, **26**, 31–38 (1995).
5) Jass, J. R., Love, S. B. and Northover, J. M. A. A new prognostic classification of rectal cancer. *Lancet*, **329**, 1303–1306 (1987).
6) Nagai, E., Yao, T., Sakamoto, M., Akazawa, K., Utsunomiya, T. and Tsuneyoshi, M. Risk factors related to liver metastasis in colorectal carcinoma: a multivariate analysis of clinicopathologic and immunohistochemical variables. *Jpn. J. Cancer Res.*, **85**, 1280–1287 (1994).
7) Suzuki, Y., Ohtani, H., Mizoi, T., Takeha, S., Shiiba, K., Matsuno, S. and Nagura, H. Cell adhesion molecule expression by vascular endothelial cells as an immune/inflammatory reaction in human colon carcinoma. *Jpn. J. Cancer Res.*, **86**, 585–593 (1995).
8) Poste, G. and Fidler, I. J. The pathogenesis of cancer metastasis. *Nature*, **283**, 139–146 (1980).
9) Danø, K., Andreasen, P. A., Grohndahl-Hansen, J., Kristensen, P., Nielsen, L. S. and Skrier, L. Plasminogen activators, tissue degradation and cancer. *Adv. Cancer Res.*, **44**, 139–266 (1985).
10) Tryggvason, K., Höyhtyä, M. and Pyke, C. Type IV collagenases in invasive tumors. *Breast Cancer Res. Treat.*, **24**, 209–218 (1993).
11) Stetler-Stevenson, W. G., Aznavoorian, S. and Liotta, L. A. Tumor cell interaction with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.*, **9**, 541–573 (1993).
12) Nakajima, M. and Chop, A. M. Tumor invasion and

extracellular matrix degradative enzymes: regulation of activity by organ factors. *Semin. Cancer Biol.*, **2**, 115–127 (1991).
13) Birkedal-Hansen, H. Role of matrix metalloproteinases in human periodontal disease. *J. Periodontol.*, **64**, 474–484 (1993).
14) Zucker, S., Lysik, R. M., Zarrabi, M. H. and Moll, U. Mr 92,000 type IV collagenase is increased in plasma of patients with colon cancer and breast cancer. *Cancer Res.*, **53**, 140–146 (1993).
15) Danø, K., Andreasen, P. A., Grohndahl-Hansen, J., Kristensen, P., Nielsen, L. S. and Skriver, L. Plasminogen activators, tissue degradation and cancer. *Adv. Cancer Res.*, **44**, 139–266 (1985).
16) Ellis, V., Pyke, C., Eriksen, J., Solberg, H. and Danø, K. The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. *Ann. NY Acad. Sci.*, **667**, 13–31 (1992).
17) Pyke, C., Ralfkiaer, E., Tryggvason, K. and Danø, K. Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. *Am. J. Pathol.*, **142**, 359–365 (1993).
18) Pyke, C., Ralfkiaer, E., Huhtala, P., Hurskainen, T., Danø, K. and Tryggvason, K. Localization of messenger RNA for Mr72,000 and 92,000 type IV collagenases in human skin cancers by *in situ* hybridization. *Cancer Res.*, **52**, 1336–1341 (1992).
19) Nielsen, B. S., Timshel, S., Kjeldsen, L., Sehested, M., Pyke, C., Borregaard, N. and Danø, K. 92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in malignant epithelial cells in human colon cancer. *Int. J. Cancer*, **65**, 57–62 (1996).
20) Pyke, C., Ralfkiaer, E., Rønne, E., Hyer-Hansen, G., Kirkeby, L. and Danø, K. Immunohistochemical detection of the receptor for urokinase plasminogen activator in human colon cancer. *Histopathology*, **24**, 131–138 (1994).
21) Matovani, A., Bottazzi, B., Colotta, F., Sozzani, S. and Ruco, L. The origin and function of tumor-associated macrophages. *Immunol. Today*, **13**, 265–270 (1992).
22) Kjeldsen, L., Bjerrum, O. W., Hovgaard, D., Johnsen, A. H., Sehested, M. and Borregaard, N. Human neutrophil gelatinase: a marker for circulating blood neutrophils. Purification and quantitation by enzyme linked immunosorbent assay. *Eur. J. Haematol.*, **49**, 180–191 (1992).
23) Michaelis, J., Vissers, M. C. and Winterbourn, C. C. Human neutrophil collagenase cleaves alpha 1-antitrypsin. *Biochem. J.*, **270**, 809–814 (1990).
24) Rønne, E., Behrendt, N., Ellis, V., Ploug, M., Danø, K. and Hoyer-Hansen, G. Cell-induced potentiation of the plasminogen activation system is abolished by a monoclonal antibody that recognizes the NH_2-terminal domain of the urokinase receptor. *FEBS Lett.*, **288**, 233–236 (1991).
25) Sorsa, T., Ding, Y., Salo, T., Lauhio, O., Teronen, T., Ingman, H., Ohtani, H., Andoh, N., Takeha, S. and Konttinen, T. Effects of tetracyclines on neutrophil, gingival, and salivary collagenases. A functional and western-blot assessment with special reference to their cellular sources in periodontal diseases. *Ann. NY Acad. Sci.*, **732**, 112–131 (1994).
26) Shin, R.-W., Iwaki, T., Kitamoto, T. and Tateishi, J. Hydrated autoclave pretreatment enhances TAU immunoreactivity in formalin-fixed normal and Alzheimer's disease brain tissues. *Lab. Invest.*, **64**, 693–701 (1992).
27) Ohtani, H., Pyke, C., Danø, K. and Nagura, H. Expression of urokinase receptor in various stromal cell populations in human colon cancer: immunoelectron microscopical analysis. *Int. J. Cancer*, **62**, 691–696 (1995).
28) Gerrard, T. L., Cohen, D. J. and Kaplan, A. M. Human neutrophil-mediated cytotoxicity to tumor cells. *J. Natl. Cancer Inst.*, **66**, 483–488 (1981).
29) Hara, I., Hotta, H., Sato, N., Eto, H., Arakawa, S. and Kamidono, S. Rejection of mouse renal cell carcinoma elicited by local secretion of interleukin-2. *Jpn. J. Cancer Res.*, **87**, 724–729 (1996).
30) Lacraz, S., Isler, P., Vey, E., Welgus, H. G. and Dayer, J. H. Direct contact between T lymphocytes and monocytes is a major pathway for induction of metalloproteinase expression. *J. Biol. Chem.*, **269**, 22027–22033 (1994).
31) Liabakk, N.-B., Talbot, I., Smith, R. A., Wilkinson, K. and Balkwill, F. Matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) type IV collagenases in colorectal cancer. *Cancer Res.*, **56**, 190–196 (1996).
32) Sitrin, R. G., Todd, R. F., Mizukami, I. F., Gross, T. J., Shollenberger, S. B. and Gyetko, M. R. Cytokine-specific regulation of urokinase receptor (CD87) expression by U937 mononuclear phagocytes. *Blood*, **84**, 1268–1275 (1994).
33) Nykjaer, A., Moller, B., Todd, R. F., Christensen, T., Andreasen P. A., Glieman, J. and Petersen, C. M. Urokinase receptor. An activation antigen in human T lymphocytes. *J. Immunol.*, **152**, 505–516 (1994).
34) Mizoi, T., Ohtani, H., Suzuki, Y., Shiiba, K., Matsuno, S. and Nagura, H. Intercellular adhesion molecule-1 expression by macrophages in human gastrointestinal carcinoma: possible roles in host immune/inflammatory reaction. *Pathol. Int.*, **45**, 565–572 (1995).
35) Chaux, P., Moutet, M., Faivre, J., Martin, F. and Martin, M. Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7-1 and B7-2 costimulatory molecules of the T-cell activation. *Lab. Invest.*, **74**, 975–983 (1996).
36) Ohtani, H., Kuroiwa, A., Obinata, M., Ooshima, A. and Nagura, H. Identification of type I collagen-producing cells in human gastrointestinal carcinomas by non-radioactive *in situ* hybridization and immunoelectron microscopy. *J. Histochem. Cytochem.*, **40**, 1139–1146 (1992).
37) Sorsa, T., Lukinmaa, P. L., Westerlund, U., Ingman, T., Ding, Y., Tschesche, H., Konittien, Y., Helaakoski, T. and Salo, T. The expression, activation and chemotherapeutic inhibition of matrix metalloproteinase-8 (neutrophil col-

lagenase/collagenase2) in inflammation. *In* "The Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants," ed. Z. Davidovitch, pp. 1–11 (1996). Harvard Society for the Advancement of Orthodontics, Boston, Massachusetts.

38) Ohtani, H., Nagai, T. and Nagura, H. Similarities of *in situ* mRNA expression between gelatinase A (MMP-2) and type I procollagen in human gastrointestinal carcinoma: comparison with granulation tissue reaction. *Jpn. J. Cancer Res.*, **86**, 833–839 (1995).

39) Gress, T. M., Muller-Pillasch, F., Lerch, M. M., Friess, H., Buhler, M. and Adler, G. Expression and *in-situ* localization of genes coding for extracellular matrix proteins and extracellular matrix degrading proteases in pancreatic cancer. *Int. J. Cancer*, **62**, 407–413 (1995).

40) Swallow, C. J., Murray, M. P. and Guillem, J. G. Metastatic colorectal cancer cells induce matrix metalloproteinase release by human monocytes. *Clin. Exp. Metastasis*, **14**, 3–11 (1996).

41) Ronne, J., Bugge, T. H., Pyke, C., Loud, L. R., Flick, M. J., Degen, J. L. and Danø, K. Impaired wound healing in mice with a disrupted plasminogen gene. *Nat. Med.*, **2**, 287–292 (1996).

42) Emmert-Buck, M. R., Roth, M. J., Zhuang, Z., Campo, E., Rozhin, J., Sloane, B. F., Liotta, A. and Stetler-Stevenson, W. G. Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. *Am. J. Pathol.*, **145**, 1285–1290 (1994).