MMP-9 Is Differentially Expressed in Primary Human Colorectal Adenocarcinomas and Their Metastases

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Abstract

Matrix metalloproteinase-9 (MMP-9) is up-regulated in macrophages in various human cancer types. In human colon cancer, MMP-9 is expressed in a macrophage subpopulation located at the tumor edge, indicating a specific induction of MMP-9 in macrophages in direct association with cancer invasion. To test whether MMP-9 is also induced in tumor edge macrophages in metastases from colorectal adenocarcinomas, we have compared the expression pattern of MMP-9 in primary colorectal adenocarcinomas (*n* = 15) with that in liver metastases (*n* = 15) and local lymph node metastases (*n* = 7) from the same patients by *in situ* hybridization and immunohistochemistry. In all the colorectal adenocarcinomas, the expression of MMP-9 mRNA and immunoreactivity in macrophages was located at the invasive front. In contrast, only 3 of the 15 liver metastases had MMP-9 mRNA and immunoreactivity at the periphery, and this expression was confined to small foci of macrophages located either among lymphocytes or in a dense desmoplastic stroma. Expression of MMP-9 mRNA and immunoreactivity was in all liver metastases seen in macrophages located in the lumen of malignant glandular structures and in central necrotic tissue. In all the 7 lymph node metastases, MMP-9 mRNA and immunoreactivity was seen in macrophages located in the stromal tissue surrounding the metastases. We conclude that MMP-9 is not up-regulated in tumor edge macrophages in liver metastases like in their primary tumor and local lymph node metastases, suggesting that disseminating colorectal cancer cells can adopt alternative proteolytic mechanisms for invasion depending on the local microenvironment. (Mol Cancer Res 2006;4(5):293–302)

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Introduction

The formation of cancer metastases is dependent on the ability of malignant cells to enter the blood and lymphatic system and subsequently form tumors in lymph nodes and distant organs. In primary adenocarcinomas, cancer cell invasion is facilitated by interaction with the local stromal compartment, whereas metastastic growth requires the ability of the cancer cell to interact with the new host tissue before the cancer cells can invade and destroy the target organ. The local stromal compartment, which comprises inflammatory cells and fibroblasts, facilitates the process by secreting extracellular matrix–degrading proteases, including matrix metalloproteinases (MMP) and urokinase-type plasminogen activator (uPA; refs. 1-5). The extracellular matrix–degrading proteases are regulated by specific extracellular inhibitors, tissue inhibitors of metalloproteinases, and plasminogen activator inhibitors as well as by cell surface receptors, such as the uPA receptor. Several extracellular matrix–degrading proteases, as well as their related activators, inhibitors, and receptors, are up-regulated in cancer tissue. For some of these components, increased levels predict a poor prognosis for the patient (1, 2, 6).

The MMPs comprise a family of zinc-dependent endopeptidases that consist of >21 human proteases. Among them, MMP-9 is secreted as a 92-kDa proenzyme, which after activation, can degrade type IV collagen and denatured collagens. Therefore, MMP-9 is also known as the 92-kDa type IV collagenase/gelatinase. This implies that MMP-9 can degrade basement membranes, which is often the initial step in carcinoma invasion. Pro-MMP-9 can be activated by plasmin *in vivo* and more efficiently by MMP-3 after plasmin-directed activation of pro-MMP-3 (7, 8). In addition, MMP-2/MMP-14 and MMP-13 can activate pro-MMP-9 (9, 10). Pro-MMP-9 is synthesized into granules in the late phase of neutrophil differentiation in the bone marrow and therefore is a normal constituent of circulating neutrophils (11), which release their granules after activation in local inflammatory tissue. Active MMP-9 is inhibited by the four known tissue inhibitors of metalloproteinases.

The tumor tissue levels of MMP-9 are elevated compared with the corresponding normal tissue in a variety of cancer types, including breast, colon, and gastric cancers (2, 4). In human colorectal adenocarcinomas, the MMP-9 mRNA and the MMP-9 plasma protein levels are also increased compared with normal individuals (12, 13). Interestingly, increased MMP-9 protein level measured in portal blood versus that measured in the peripheral blood has been reported to predict liver

metastasis (14). By histologic localization studies, we and others have found previously that MMP-9 mRNA and/or protein are induced in a small subpopulation of macrophages located at the invasive front in the vicinity of the invasive cancer cells, suggesting that the MMP-9-expressing macrophages are important for the invasion of cancer cells into the normal tissues (15-17). Indeed, in a murine skin cancer model, the lack of MMP-9 expressed by bone marrow–derived cells decreased the number of malignant lesions and modified the degree of malignancy (18).

To investigate whether the particular expression of MMP-9 in the invasive front of human colorectal adenocarcinomas is also an active part of the behavior of their metastasis, we have compared the histologic expression pattern of MMP-9 in primary colorectal adenocarcinomas with that in their metastasis to the liver and lymph nodes of the same patients. We show that in contrast to the primary tumors MMP-9 is only sporadically induced in macrophages at the invasive periphery of the liver metastases despite a substantial stromal response.

Results

In this study, 15 primary colorectal adenocarcinomas, 15 liver metastases, and 7 samples with local lymph node metastases from the same patients were analyzed. The tumor edge of the primary lesions was categorized according to the presence of single cell invasion (budding) and the liver metastases according to their growth pattern: inducing desmoplasia, solid, or replacing growth (see Materials and Methods).

MMP-9 mRNA Expression

Primary Colorectal Adenocarcinomas. The 15 samples of human colon cancer and their matched samples from liver and local lymph node metastases were analyzed for the presence of MMP-9 mRNA by *in situ* hybridization using ^{35}S-labeled MMP-9 riboprobes. MMP-9 mRNA was detected in all 15 primary colorectal adenocarcinomas and was seen in stromal cells identified as macrophages (see below) at the invasive edge (see Fig. 1A and D). The primary cancers with prevalent cancer cell budding showed a relatively higher number of MMP-9

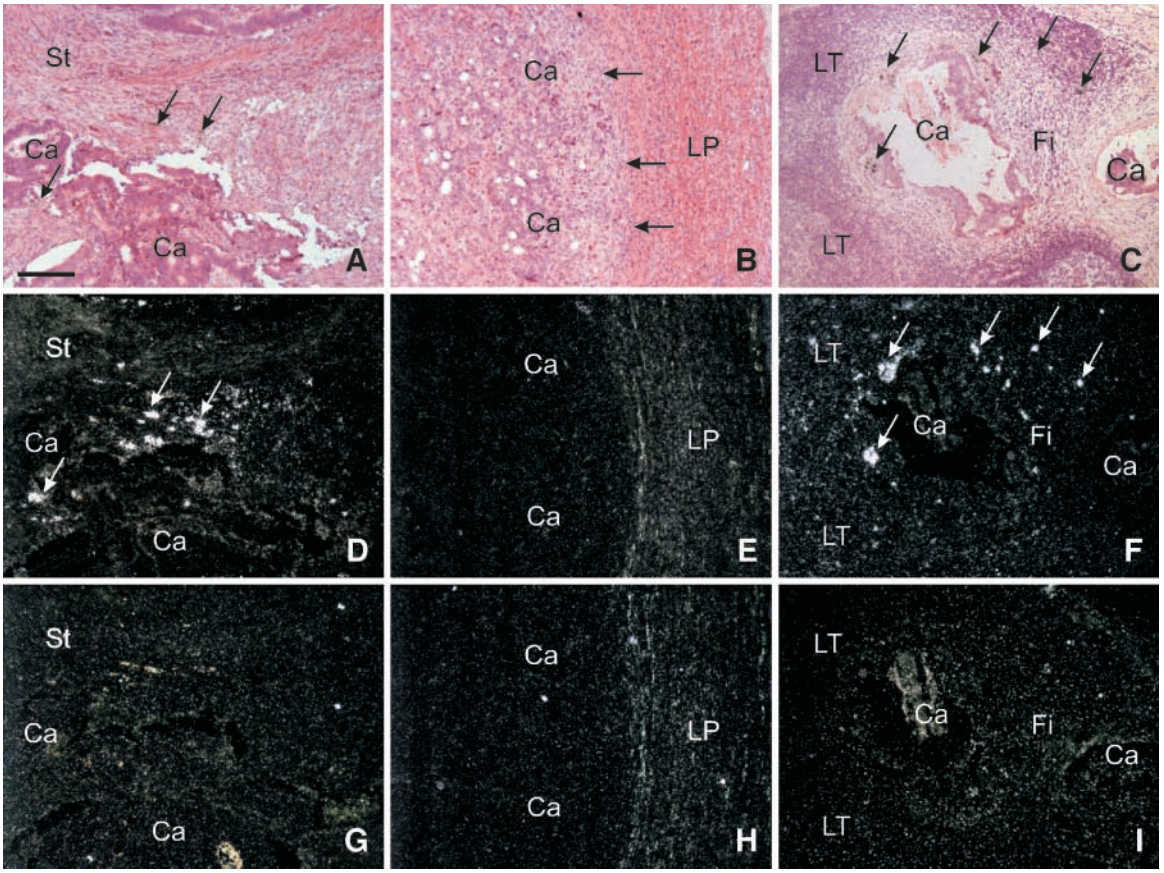

FIGURE 1. *In situ* hybridization for MMP-9 mRNA in a primary colon adenocarcinoma and its metastases to the liver and a local lymph node. Two serial sections from a primary colon cancer (**A, D**, and **G**), a liver metastasis (**B, E**, and **H**), and a lymph node metastasis (**C, F**, and **I**) from the same patient (case 3 in Table 1) were processed for MMP-9 mRNA *in situ* hybridization with either a ^{35}S-labeled antisense (**A-F**) or the corresponding sense probe (**G-I**). The MMP-9 mRNA signal is visualized as black silver grains in bright-field illumination (**A-C**) and as white spots in dark-field illumination (**D-I**). In the primary cancer, the MMP-9 mRNA is seen in stromal cells (**A** and **D**, *arrows*) in the submucosa (*St*) at the invasive edge of the cancer (*Ca*). In the liver metastasis, no MMP-9 mRNA is seen at the invasive front of the cancer (**B** and **E**). Arrows in (**B**) indicate the interface separating the cancer cells from the liver parenchyma (*LP*). In the local lymph node metastasis, the MMP-9 mRNA is seen in stromal cells (**C** and **F**, *arrows*) in fibrotic tissue (*Fi*) surrounding the cancer cells. Only a few MMP-9 mRNA-expressing cells can in this case be identified in the lymphatic tissue (*LT*). No *in situ* hybridization signal is seen with the MMP-9 mRNA sense probe (**G-I**). Bar, ~200 μm.

Table 1. Expression of MMP-9 mRNA and Immunoreactivity in Tumor Edge Macrophages in 15 Primary Colorectal Cancers and Their Metastases

Case	Primary colorectal cancers		Liver metastasis		Lymph node metastasis	
	mRNA	Immunoreactivity	mRNA	Immunoreactivity	mRNA	Immunoreactivity
1	+	+	–	–	+	+
2	+	+	+*	+*	NA†	NA
3	+	+	–	–	+	+
4	+	+	+*	+*	NA	NA
5	+	+	–	–	+	+
6	+	+	–	–	NA	NA
7	+	+	–	–	+	+
8	+	+	–	–	+	+
9	+	+	+*	+*	NA	NA
10	+	+	–	–	+	+
11	+	+	–	–	NA	NA
12	+	+	–	–	+	+
13	+	+	–	–	NA	NA
14	+	+	–	–	NA	NA
15	+	+	–	–	NA	NA

*Expression in small foci.
†NA, lymph nodes not available.

mRNA-positive tumor edge macrophages than lesions with low local invasive activity. We also observed scattered MMP-9 mRNA-positive macrophages in central stromal areas in 5 cases and in macrophages located in the lumen of some of the tumor glands in 11 of the 15 samples (data not shown).

Liver Metastases. MMP-9 mRNA was detected in all the 15 metastases. In 3 of the cases (2, 4, and 9; see Table 1), a MMP-9 mRNA signal was seen in macrophages in small stromal foci at the metastasis periphery; the MMP-9 mRNA-positive macrophages were associated with desmoplasia in 1 case and with occasional accumulation of lymphocytes in the other 2 cases (Table 1). The remaining 12 liver metastases were without detectable MMP-9 mRNA in the tumor edge (Fig. 1B and E). However, all the 15 cases had strong MMP-9 mRNA signal in macrophages located in central areas (see Fig. 2H and I) and in macrophages located within the lumen of the malignant colon epithelium (data not shown). In 1 case (7), which was the only metastasis showing the replacement growth pattern, we observed MMP-9 mRNA expression in cells associated with vessels (data not shown). No MMP-9 mRNA signal was seen in the adjacent unaffected normal liver tissue in any of the 15 cases.

Lymph Node Metastases. Paraffin blocks from 7 patients containing a total of 23 lymph nodes (range, 1-6) with colon cancer metastasis in 10 of these (range, 1-3) were available for the study. The tumor metastases were in 6 patients (9 metastases) surrounded by both desmoplasia and lymphatic tissue and in one patient (1 metastasis) by lymphatic tissue only. MMP-9 mRNA was detected in all lymph nodes with metastasis. The MMP-9 mRNA was observed in macrophages, which in all the former 6 patients were located in the desmoplastic margin (Fig. 1C and F; Table 1) and in the latter patient in the adjacent lymphatic tissue. MMP-9 mRNA-positive macrophages were seen in 3 patients (4 metastases) within malignant crypt lumens or in central necrotic areas. Surprisingly MMP-9 mRNA was also found in 7 of the lymph nodes (from 3 patients) without evident metastatic cells, whereas no signal was detected in the remaining metastasis-negative lymph nodes.

Control Experiments. For all samples, two nonoverlapping MMP-9 antisense probes were applied on adjacent sections. In all cases, these showed an identical hybridization pattern. In all cases, the corresponding sense probes were used as negative controls and showed only background signal (Fig. 1G-I).

MMP-9 Immunoreactivity

Primary Colorectal Adenocarcinomas. MMP-9 immunoperoxidase staining was done on all samples using affinity-purified rabbit polyclonal antibodies against MMP-9. In all 15 cases, MMP-9 immunoreactivity was detected in macrophages located at the tumor edge (Fig. 2A). In 5 of the cases, we also observed scattered MMP-9-positive macrophages within the central tumor stroma. These 5 cases were the same as those in which the MMP-9 mRNA was detected in macrophages with a similar location (see above). In all colon cancer lesions, MMP-9-positive macrophages were also seen within the lumen of some of the malignant crypts. MMP-9 immunoreactive polymorphonucleated cells (neutrophils) were observed throughout the resected tissue and were particularly numerous at the invasive front of lesions with prevalent single cell invasion. In some areas, MMP-9 immunoreactive neutrophils also invaded and occupied the malignant crypts when tumor cells detached from the glandular structures leading to opened lumina (data not shown). Except from neutrophils, no MMP-9 immunoreactive cells were observed in the normal unaffected colon tissue present in the samples. We have previously found MMP-9 immunoreactivity in macrophages within small local lymphatic foci adjacent to normal colon mucosa (Peyer's patches) present in samples with colorectal cancer (16). These foci were not present in the current colon material because the tissue samples were obtained from the profound tumor edge.

Double immunofluorescence analyses were done on all colon cancer samples with the MMP-9 antibodies together with a macrophage specific antibody (CD68, clone PG-M1). In all

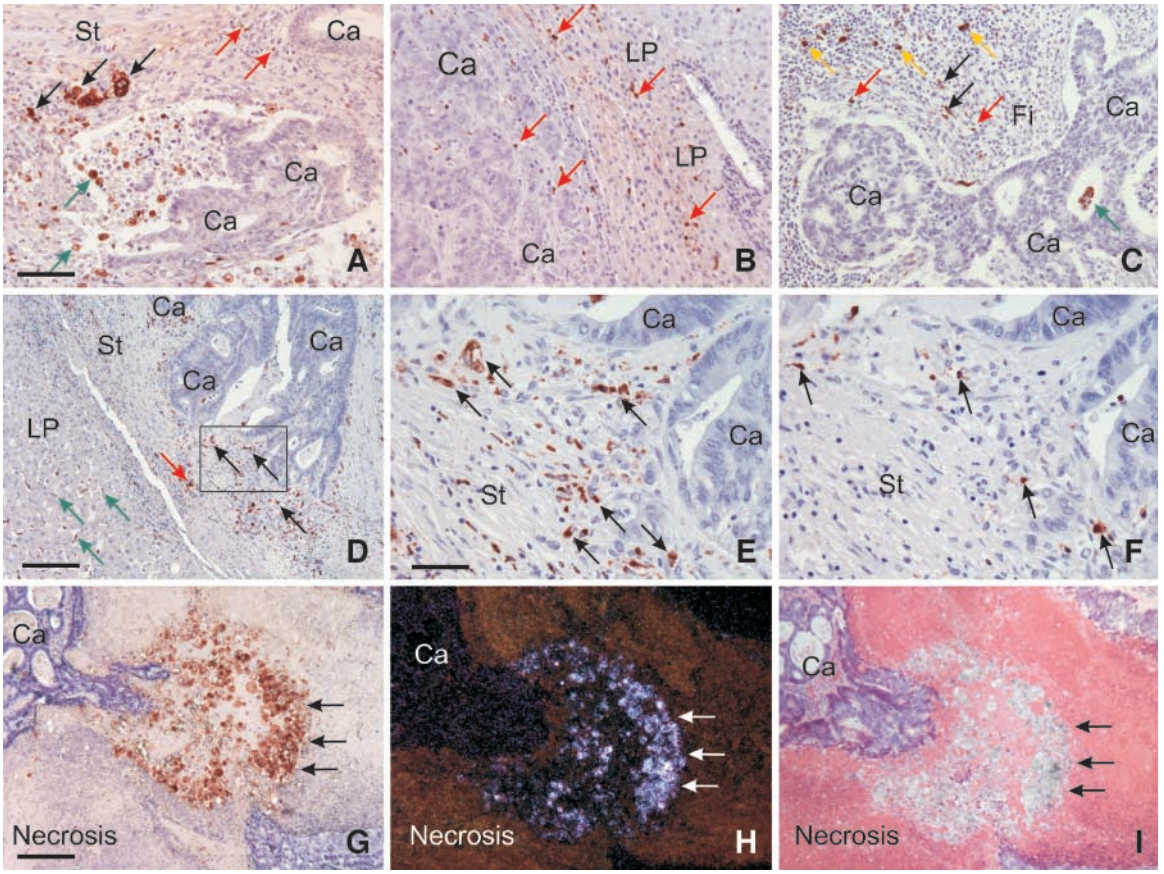

FIGURE 2. Immunoperoxidase staining for MMP-9 in a primary colon adenocarcinoma and its metastases to the liver and a local lymph node, and comparison with MMP-9 mRNA expression in a liver metastasis. Sections from tissue samples obtained from the same patient (case 3) primary colon cancer (**A**), liver metastasis (**B**), and local lymph node metastasis (**C**) were processed for immunohistochemistry using affinity-purified rabbit polyclonal antibodies against MMP-9. In the primary colon cancer, the MMP-9 immunoreactivity is seen in macrophage-like cells (**A**, *black arrows*) located in the stroma adjacent to the invasive cancer cells, in macrophage-like cells located within tumor glands (**A**, *green arrows*), and in polymorphonucleated cells (**A**, *red arrows*). In the liver metastasis, MMP-9 immunoreactivity is seen in numerous polymorphonucleated cells (**B**, *red arrows*) located in the stroma of the metastasis, here in the interface between cancer cells and the liver parenchyma. In the local lymph node metastasis, MMP-9 immunoreactivity is seen in stromal macrophage-like cells (**C**, *black arrows*) located in an area of fibrosis. Note also the MMP-9-positive macrophage-like cells within the lymphatic tissue (**C**, *yellow arrows*), neutrophils (**C**, *red arrow*), and intraluminal macrophages (**C**, *green arrow*). Two neighboring sections from a liver metastasis were processed for immunohistochemistry using a monoclonal antibody to CD68 (**D** and **E**) and polyclonal antibodies to MMP-9 (**F**). $CD68^+$ macrophages are seen especially in the stroma at the tumor edge (**D** and **E**, *black arrows*; *box* in **D** corresponds to close-up in **E** an **F**), in the fibrotic area (**D**, *red arrow*), and in the liver parenchyma (as Kupffer cells; **D**, *green arrows*). In the adjacent section, MMP-9 immunoreactivity is only seen in polymorphonucleated cells in the metastasis periphery (**F**, *black arrows*). Two sections of a liver metastasis were processed either for MMP-9 immunoperoxidase staining (**G**) or for MMP-9 mRNA *in situ* hybridization (dark-field illumination in **H** and bright-field illumination in **I**). MMP-9 immunoreactivity is seen in macrophage-like cells in necrotic tissue (**G**, *arrows*) adjacent to live cancer cells. This pattern is identical to the MMP-9 mRNA expression pattern (**H** and **I**, *arrows*). Bar, ~100 μm (**A-C**), ~400 μm (**D**), ~50 μm (**E** and **F**), and ~200 μm (**G-I**).

cases, all the MMP-9-positive cytoplasm-rich mononuclear cells were $CD68^+$ (Fig. 3A-C). Only a small fraction of the total population of $CD68^+$ macrophages located at the tumor edge was, however, MMP-9 positive (Fig. 3B). Double immunofluorescence analyses with an antibody against cytokeratin (CK) showed no coexpression in any of the cases, indicating that all carcinoma cells in all cases were MMP-9 negative (data not shown).

Liver Metastases. MMP-9 immunoperoxidase-stained macrophages and neutrophils were present in all cases. At the periphery of the metastases, only 3 of the 15 cases (cases 2, 4, and 9; Table 1) had MMP-9-positive macrophages, concurrent with the *in situ* hybridization results. In the periphery of the remaining 12 liver metastases, MMP-9 immunoreactivity was only observed in neutrophils (Fig. 2B) despite the presence of $CD68^+$ macrophages in all the 12 cases, particularly numerous in metastases with a prevalent desmoplastic growth pattern (Fig. 2D-F). Intense MMP-9 immunoreactivity was seen in 14 of the liver metastases in macrophages within centrally located necrotic tissue (Fig. 2G) and in macrophage-like cells intraluminally in some malignant crypts (data not shown). In one liver metastasis (case 7), MMP-9 immunoreactivity was seen in vascular cells located in a hypervascularized and strongly fibrotic area (data not shown). In the normal liver tissue, scattered MMP-9-positive neutrophils were observed, but no MMP-9 immunoreactivity was seen in Kupffer cells, liver parenchyma, or vessels. Double immunofluorescence analyses, combining the polyclonal MMP-9 antibodies with the monoclonal CD68 antibody, showed the presence of MMP-9 in intraductal and necrosis-associated $CD68^+$ macrophages (see

Fig. 3D-F) in all 14 cases where these cell populations were present. $CD68^+$ macrophages located at the tumor edge were generally MMP-9 negative, except from the 3 cases described above in which small subpopulations of the $CD68^+$ macrophages were also MMP-9 positive. All $CD68^+$ macrophages (Kupffer cells) found in the normal liver tissue were MMP-9 negative. The MMP-9-positive vascular cells observed in 1 case as well as the MMP-9-positive granulocytes were $CD68^-$. MMP-9 and neutrophil elastase double immunofluorescence showed consistent colocalization of all MMP-9-positive cells located in the tumor edge of three metastases tested (cases 5, 13, and 15). No colocalization of MMP-9 with CK immunoreactivity was found in any of the metastases (data not shown).

Lymph Node Metastases. MMP-9 immunoreactivity was seen in macrophage-like cells located in areas of metastasis-induced desmoplasia or in adjacent lymphatic tissue in all 10 lymph nodes (from 7 patients) with histologic detectable metastases (Fig. 2C). In addition, MMP-9-positive macrophage-like cells were found inside crypt lumens and in necrotic tissue in the lymph node metastasis. Irrespective of the metastatic foci, MMP-9 immunoreactivity was with varying staining intensity seen in scattered macrophage-like cells located in the sinusoids throughout the lymphatic tissue in all the 23 lymph nodes. All MMP-9-positive macrophage-like cells were found to be $CD68^+$ by double immunofluorescence. A few MMP-9-positive neutrophils were observed in virtually all the lymph nodes.

Control Analyses. To test the specificity of the immunohistochemical staining of MMP-9, two adjacent sections from two primary tumors, two liver metastases, and two lymph node metastases were stained in different ways. We thus incubated the sections with the rabbit polyclonal antibodies to MMP-9 raised against MMP-9 from human neutrophils (used for the immunohistochemical staining described above) and a preparation of rabbit polyclonal antibodies raised against a MMP-9-specific peptide sequence (see Materials and Methods), respectively. The two antibody preparations gave in each of the 6 cases an identical staining pattern (results not shown). As a negative control in the immunohistochemical analyses, we replaced the rabbit polyclonal antibodies against MMP-9 with normal rabbit immunoglobulin. No staining was seen in any of the 6 samples tested, indicating that there was no nonspecific binding of rabbit IgG to the tissue sections and that no endogenous peroxidase was active (results not shown). We also preincubated the polyclonal antibodies against neutrophil MMP-9 with a 10-fold molar excess of recombinant human

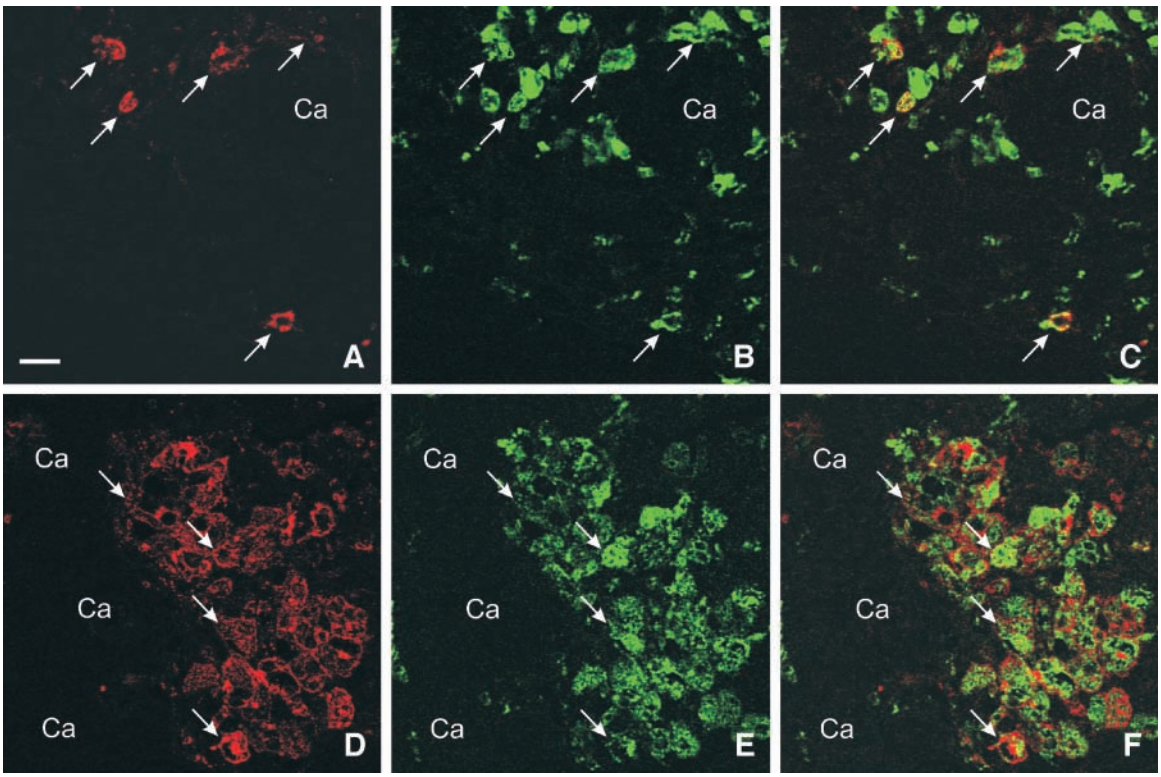

FIGURE 3. Double immunofluorescence histochemistry for MMP-9 and CD68 in a primary colorectal adenocarcinoma and its liver metastasis. Tissue sections from a primary colon adenocarcinoma (**A-C**) and a liver metastasis (**D-F**) from the same patient (case 6 in Table 1) were incubated with rabbit polyclonal antibodies against MMP-9 together with a mouse monoclonal antibody against CD68. The area in (**A-C**) is from the tumor edge of a colon adenocarcinoma similar to the area shown in Fig. 2A. The area in (**D-F**) is showing a necrotic area in the liver metastasis similar to that illustrated in Fig. 2G. The polyclonal antibodies were detected with Cy3-labeled goat anti-rabbit IgG (*red fluorescence* in **A, C, D**, and **F**) and the monoclonal antibody with FITC-labeled goat anti-mouse IgG (*green fluorescence* in **B, C, E**, and **F**). Colocalization of the two fluorophores is displayed in yellow (**C** and **F**). All of the MMP-9-positive cells in these images (**A, C, D**, and **F**, *arrows*) are positive for CD68 (**B, C, E**, and **F**, *arrows*). At the edge of colorectal adenocarcinoma, a subpopulation of the $CD68^+$ cells are MMP-9 positive (**B** and **C**), whereas virtually all $CD68^+$ cells in the necrotic area are MMP-9 positive (**E** and **F**). Bar, ~40 μm.

MMP-9, which in all 6 cases led to the elimination of the MMP-9 immunoreactivity in the various cell populations identified above. Direct comparison of MMP-9 immunohistochemical staining and MMP-9 mRNA *in situ* hybridization was also done on serial sections of these primary tumors, liver metastases, and local lymph node metastases. By aligning the serial sections, we observed that all areas with cells showing MMP-9 *in situ* hybridization signal were found also to have MMP-9 immunoreactivity in macrophage-like cells (Fig. 2G-I) in all of 6 samples analyzed. This analysis also showed that MMP-9-positive neutrophils were not accompanied by MMP-9 mRNA. This is in accordance with MMP-9 being stored in these cells (11) and also indicates that possible *de novo* synthesis of MMP-9 mRNA in these cells does not occur or is under the detection limit of the method.

MMP-9 Expression in Primary Liver Tumors

We also studied the MMP-9 expression in primary liver carcinomas, 10 hepatocellular and 11 cholangiocarcinomas, by immunohistochemistry. Numerous $CD68^+$ macrophages were present in all 21 samples, but in only 1 of the 10 hepatocellular carcinomas and in 4 of the 11 cholangiocarcinomas we observed foci with MMP-9-positive macrophages in the desmoplastic tumor edge. Scattered MMP-9-positive neutrophils were seen in all 21 cases, and in 2 of the hepatocellular carcinomas and 4 cholangiocarcinomas, we observed MMP-9-positive elongated spindle-shaped cells, which were directly associated with vessels and were considered to be vascular pericytes, because they often showed an abluminal localization and resembled MMP-9-positive vascular cells present in human breast cancer, which we have identified previously as pericytes (19).

Discussion

Invasion of colorectal cancer cells into the neighboring tissues is generally accompanied by pronounced activation of its stromal surroundings, leading to inflammation, desmoplasia, and neovascularization. The cancer cells together with the accompanying stromal cells form a complex cellular community that invades the normal tissue facilitated by increased proteolytic activity. In this study, we have shown, by comparison of primary colon cancers and their matched liver metastases, that tumor edge macrophages in the metastases rarely express MMP-9 in contrast to the consistent expression of MMP-9 in tumor edge macrophages in their colon cancer of origin. This finding suggests that the disseminating colon cancer cells for invasion of the liver tissue use alternative proteolytic mechanisms, which are determined by the local cellular and extracellular environment.

Our study is, to the best of our knowledge, the first to compare the histologic expression patterns of any MMP in matched primary colon cancers and their liver metastases and was based on a combination of MMP-9 mRNA *in situ* hybridization analyses and MMP-9 immunohistochemistry. Two nonoverlapping MMP-9 *in situ* hybridization probes showed an identical hybridization pattern and sense probes used as negative controls resulted in no specific signal. There was a good correlation between the localization of the MMP-9 mRNA and that of the MMP-9 immunoreactivity in mononuclear cells; the polymorphonucleated cells expressed MMP-9 protein only, in accordance with its synthesis in the bone marrow (20). In addition, comparison of the MMP-9 immunoreactivity obtained by two preparations of polyclonal antibodies raised against neutrophil MMP-9 and a MMP-9 peptide, respectively, showed an identical immunohistochemical staining pattern. Nonimmune rabbit immunoglobulin showed no staining, and preabsorption of the MMP-9 antibody with recombinant protein abolished the staining of the various cell types. We conclude therefore that the MMP-9 *in situ* hybridization signal and MMP-9 immunoreactivity detected represent genuine MMP-9 mRNA and protein, respectively.

Expression of MMP-9 mRNA and protein in macrophages in the liver metastases was not generally suppressed because MMP-9 mRNA and protein were observed in macrophages accumulating inside some of the tumor glands as well as in numerous macrophages associated with central necrosis in all the investigated liver metastases. These, MMP-9-expressing macrophages were remote from the adjacent liver tissue and could therefore not be directly involved in the invasion process. Importantly however, the MMP-9 mRNA and immunoreactivity in these cells served as a unique internal positive control for the *in situ* hybridization and the immunoperoxidase staining in the individual metastases and thereby strongly substantiated the validity of our findings.

The MMP-9 mRNA and immunohistochemical expression pattern observed in the primary colorectal carcinomas substantiates our previous studies on the localization MMP-9 mRNA and immunoreactivity in colorectal cancer tissue specimens done on other patient groups (15, 16) and is in agreement with MMP-9 immunohistochemical and *in situ* hybridization studies reported by others (12, 21). Our findings on colon cancer liver metastases, however, partly disagree with results reported by Zeng et al. (12), who analyzed MMP-9 mRNA expression in colorectal cancer and 10 unmatched samples of colon cancer liver metastases. They employed an *in situ* hybridization approach that seems to have been quite similar to the one we have used but found MMP-9 mRNA up-regulated in macrophages at the tumor/liver interface. We cannot explain this difference, but it is noteworthy that in contrast to our findings they did not report any MMP-9 mRNA expression in necrotic areas or within the lumen of tumor glands, suggesting major differences in our methods or observation materials.

Single cancer cells or small clusters of cancer cells from invasive colorectal adenocarcinomas can disseminate to local lymph nodes as well as enter the circulation and reach distant organs, including the liver. In the liver, the colon cancer cells invade the normal liver parenchyma following either of two major pathways. In ~50% of the cases, the presence of cancer cells results in recruitment of local stromal cells, including fibroblasts and macrophages, leading to extensive desmoplasia (22). In the other half, the malignant cells invade the liver tissue with a solid growth pattern, showing no remarkable reaction in the surrounding stromal compartment or any increase in inflammatory cells. In this study, we included 15 cases of colon cancer liver metastasis, 8 of which showed extensive tumor edge desmoplasia with numerous macrophages, but in only 2 of them and in 1 metastasis with the solid growth pattern

we observed small single foci, where MMP-9 (mRNA and protein) was detected in macrophages at the tumor edge. Thus, in contrast to its primary lesions, the invasive periphery of the liver metastasis is generally not accompanied by the expression of MMP-9 (mRNA and protein) in macrophages. Whether other MMPs show similar discrepancies in their expression patterns in primary colorectal tumors versus their liver metastases remains to be determined.

We also analyzed the expression of MMP-9 in local lymph node metastases from 7 of the colon cancer patients. Similar to the MMP-9 mRNA and immunohistochemical expression patterns in the primary tumors, we observed MMP-9 mRNA and immunoreactivity in tumor edge macrophages in all 7 patients. In the lymphatic tissue present in the lymph node metastases, we interestingly observed MMP-9 mRNA and protein in some scattered macrophages. We consider this expression pattern analogous to the expression of MMP-9, which we have found previously in Peyer's patches in samples of primary colon cancer tissue (16). In addition, in all 13 lymph nodes without apparent metastasis from the 7 patients, we saw MMP-9 immunoreactivity in scattered macrophages. It remains to be clarified whether this MMP-9 expression is related to the neighboring colon cancer.

Several other extracellular matrix–degrading proteases, their receptors, and inhibitors are highly up-regulated in primary human colorectal cancer, including many MMPs and tissue inhibitors of metalloproteinases and components of the plasminogen activation system (23-27). Many of these molecules are mainly expressed by stromal cells, such as fibroblasts, macrophages, and endothelial cells. Stromal cell expression of components of protease systems is also seen in other types of cancer. The cellular expression pattern of these molecules is, however, often different in different types of primary cancers (28, 29). In primary colon cancers, we thus have, in addition to the macrophage expression of MMP-9, found previously that both uPA and plasminogen activator inhibitor-1 are expressed by fibroblasts and plasminogen activator inhibitor-1 also by endothelial cells but neither of the two molecules by the cancer cells (27, 30). In contrast, MMP-9, uPA, and plasminogen activator inhibitor-1 are all expressed by the cancer cells in primary squamous cell skin cancer and MMP-9 in addition by macrophages (31-33). Our present findings indicate that the pattern of protease expression differs not only according to the type of the primary cancer but also according to the type of tissue in which the cancer cells eventually settles. This adds another level of complexity to the stromal cell involvement in the generation and regulation of proteolysis in cancer invasion.

We show that MMP-9 is up-regulated in a small subpopulation of $CD68^+$ macrophages in the edge of primary tumors, whereas the great majority of macrophages are devoid of any MMP-9 mRNA or immunoreactivity. MMP-9 is absent in unstimulated monocytes (34), whereas it often is expressed by macrophages in relation to normal and pathologic tissue remodeling processes, probably reflecting specific stimulation. MMP-9 is thus expressed in the specialized macrophage-like cells, osteoclasts, during normal bone development (35) and in macrophages and neutrophils in rheumatoid arthritis (36). MMP-9 is induced in macrophages *in vitro* by cytokines or growth factors, including tumor necrosis factor-α, interleukin-1β, and granulocyte macrophage colony-stimulating factor (37), but the molecular mechanisms regulating the expression of MMP-9 in macrophages *in vivo* are not known and may vary from tissue to tissue. Tumor necrosis factor-α is induced in a subpopulation of macrophages in chronically inflamed liver tissue (38). Notably, its effect on MMP-9 expression in macrophages is suppressed by transforming growth factor-β (39), and the transforming growth factor-β expression level is increased in metastatic colon cancer cells compared with that in their colon cancer from which they originate (40), which may help to explain the lack of MMP-9 expression in the tumor edge macrophages in the liver metastasis.

The focal and intense MMP-9 expression at the tumor edge of colorectal cancers may suggest a promoting role of macrophage- and neutrophil-derived MMP-9 in invasion and thereby in the first step of liver metastasis. An experimental evaluation of this question would require animal models of colon cancer, which mimics this expression and metastasize to the liver. Colon cancer metastasis has been reported in several xenografted transplantation models in mice (41). In these models, MMP-9 may come from the human carcinoma cells themselves (42) and/or from the host stromal cells (43). Several genetically induced colon tumor models have been reported (41, 44) of which a few progress to an invasive stage (45-47), but none of them metastasize to the liver. The role of MMP-9 derived from inflammatory cells has, however, been studied in a transgene human papillomavirus skin cancer model in mice (18). In this model, MMP-9 is primarily expressed by macrophages, neutrophils, and mast cells. Genetic inactivation of the MMP-9 gene was found to lead to a decreased incidence of invasive tumors, which was restored by transplantation of bone marrow cells from wild-type mice, indicating that MMP-9 derived from these cells contribute to carcinogenesis in this model (18). A role of a stromal cell produced protease has also been shown in breast cancer genetically induced by the polyoma middle T antigen in mice. In this model, uPA is, like in human breast cancer, expressed by fibroblasts and uPA deficiency leads to a decrease in lung metastasis (48). In that study, we interestingly found uPA mRNA expressed in single stromal cells in the periphery of lung metastases, suggesting that the uPA expression pattern here is recapitulated in the lung tissue. In addition, macrophages have been implicated in metastasis in the polyoma middle T model, but it is not known whether this is related to their production of proteases (49).

Metastasis is a multistep process in which the cancer cells initially invade the neighboring tissue and escape from the primary tumor and ultimately through the target organ. The present study suggests that invasion involves different proteolytic mechanisms at different steps of the metastatic process.

Materials and Methods

Tissue Samples

Samples from 15 colorectal adenocarcinomas (11 Dukes D and 4 Dukes C) were obtained together with 11 synchronous and 4 metachronous liver metastases. There was a mean of 533 days between the first operation and the subsequent detection of metastasis (median, 488 days) in those with metachronous

disease. The liver metastases were removed during partial hepatectomy with a mean interval of 197 days (median, 129 days; range, 34-781 days) after the discovery of metastasis. Following surgical resection, ~4-mm-thick tissue specimens were on the same day dissected from the tumors and immediately thereafter fixed in 4% neutral-buffered formalin for 20 to 24 hours at room temperature and then paraffin embedded. The sample group comprised 6 women and 9 men in the age interval 49 to 79 years (age at colon surgery). From 7 of the patients, a single paraffin block containing local lymph node metastases was obtained. A total of 23 lymph nodes, 10 with apparent metastases, were present in these paraffin blocks. The paraffin samples with liver metastases were collected prospectively between 1997 and 1999 and their matched primary tumors and lymph nodes retrieved from the pathology archive at the Royal Hallamshire Hospital (Sheffield, United Kingdom). Specimens from primary tumors, which contained the invasive periphery, were included. Their use for research had been approved by the South Sheffield Research Ethics Committee (SS/00/342).

To ascertain that the liver biopsies contained colon metastases and were not primary liver tumors, immunoperoxidase staining (see below) of CK20 (principal marker of epithelial cells of colorectal origin), hepatocyte antigen (marker of liver cells), and CK7 (marker of bile ducts and bile duct–related cancers) of the colon and liver samples were done (50). All 15 liver lesions had $CK20^+$ tumor cells and were negative for CK7 and hepatocyte antigen. Immune reaction for CK7 was seen in bile ducts and the hepatocyte antigen in liver parenchyma.

The growth patterns of the tumors in the 15 liver metastases were divided into three groups according to the reticulin staining pattern in combination with α-smooth muscle actin and CD34 immunohistochemistry: a *desmoplastic* growth pattern, solid tumor invasion (or a *pushing* growth pattern), and a *replacement* growth pattern (22, 51). Of the 15 liver metastases, 8 showed prevalent desmoplastic stroma formation, 6 showed general solid invasion, and only 1 showed a predominant replacement growth pattern. CK20 immunohistochemistry was also used to evaluate local cancer cell invasion at the invasive front of the colon biopsies. Invasion of single cancer cells or small cell clusters containing fewer than five cancer cells is known as "budding" (52). The colorectal adenocarcinomas were divided into two groups according to their degree of cancer cell budding [none to mild budding (BD1; $n = 6$) and moderate to severe budding (BD2; $n = 9$)] as described by Tanaka et al. (53). Comparing the growth patterns in the liver metastases (desmoplastic, solid, or replacement) with that in the primary tumors (BD1 or BD2) revealed no obvious correlations.

Ten paraffin-embedded samples with hepatocellular carcinoma from 4 women and 6 men with an age range of 44 to 81 years as well as 11 paraffin-embedded samples with cholangiocarcinomas from 7 women and 4 men with an age range of 23 to 76 years were collected from Royal Hallamshire Hospital. In all the hepatocellular carcinomas, the cancer cells were positive for hepatocyte antigen and negative for CK7, whereas in the cholangiocarcinomas the cancer cells were negative for hepatocyte antigen and positive for CK7.

Antibodies

Affinity-purified rabbit polyclonal antibodies against human MMP-9 were a generous gift from Niels Borregaard (Department of Hematology, Rigshospitalet, Copenhagen, Denmark) and have been described previously by Kjeldsen et al. (20). A preparation of rabbit polyclonal antibodies directed against the amino acid sequence LGRFQTFEGDLKWH (146-159, accession no. J05070) of the MMP-9 protein was a generous gift from Majken Nielsen (Dako, Glostrup, Denmark). The Ig fraction was affinity purified against the peptide. Monoclonal antibodies against pan-CK (clone AE1/AE3), CK7 (clone OV-TL 12/30), CK20 (clone K_s20.8), α-smooth muscle actin (clone 1A4), CD34 (clone QBEnd 10), CD68 (clone PG-M1), hepatocyte (clone OCH1E5), neutrophil elastase (clone NP57), and normal rabbit immunoglobulin; FITC-conjugated goat anti-mouse IgG; and Envision reagents [which include conjugated polymers of horseradish peroxidase and goat anti-rabbit IgG (K4003) or goat anti-mouse IgG (K4001)] were all obtained from Dako. Cy3-conjugated goat anti-mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA).

Immunoperoxidase Staining

Paraffin sections were cut 3 μm thick and deparaffinized with xylene and hydrated through ethanol and water solutions. Antigen retrieval for MMP-9, CD68, CK7, CK20, pan-CK, and rabbit Ig was done by proteinase K digestion (5 μg/μL) for 20 minutes. Antigen retrieval for α-smooth muscle actin, CD34, hepatocyte, and the MMP-9 peptide antibody was done with heat induction at 98°C for 20 minutes in a Micromed microwave (Milestone, Sorisol, Italy). After pretreatment, the sections were blocked for endogenous peroxidase activity by incubation in 1% hydrogen peroxide for 15 minutes and washed briefly in TBS [50 mmol/L Tris, 150 mmol/L NaCl (pH 7.6)] containing 0.5% Triton X-100. The primary antibodies were incubated at the following concentrations or dilutions: MMP-9 (0.6 μg/mL), CD68 (1:200), CD34 (1:400), CK7, CK20, α-smooth muscle actin, pan-CK, and hepatocyte (1:300), and detected with Envision reagents as described (27). Negative controls included preabsorption with a 10-fold molar excess of purified recombinant soluble human MMP-9 protein (Oncogene, San Diego, CA) for 60 minutes at room temperature before application to the tissue sections or substitution of the polyclonal MMP-9 IgG by normal rabbit immunoglobulin.

Double Immunofluorescence

Double immunofluorescence histochemistry was done essentially as described by Illemann et al. (27). All sections were pretreated with proteinase K as specified above. Briefly, the MMP-9 polyclonal antibodies (0.6 μg/mL) were diluted in TBS-bovine serum albumin with mouse monoclonal antibodies against a CK mixture [CK20 (1:200), pan-CK (1:300), CK7 (1:200), CD68 (1:25), α-smooth muscle actin (1:200), CD34 (1:400), and neutrophil elastase (1:100)]. The polyclonal MMP-9 antibodies were detected with Cy3-conjugated goat anti-rabbit IgG (1:200) and the mouse monoclonal antibody with FITC-conjugated goat anti-mouse IgG (1:200). The double-stained sections were analyzed using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Jena, Germany)

equipped with a 488 nm argon laser and a 543 nm He-Ne laser. The images were obtained using the λ mode (pinhole diameter, 135 μm) collecting images from 509 to 595 nm wavelength. For separation of the specific fluorescence signals, we first obtained FITC, Cy3, and erythrocyte autofluorescence emission spectra from single fluorophore-stained sections (for FITC or Cy3 fluorescence) or unstained sections (for erythrocyte autofluorescence). From double-labeled sections, the collected fluorescence signal was separated by the emission fingerprinting using the above emission spectra to separate the individual fluorescence signals as described (54).

In vitro *Transcription*

^{35}S-labeled antisense and sense MMP-9 riboprobes were generated from plasmids pCOL9201 and pCOL9202 (32) by incorporation of ^{35}S-labeled UTP (NEN, Boston, MA) by *in vitro* transcription using T3, T7, and SP6 RNA polymerase (Roche, Basel, Switzerland). The DNA template was digested with DNase (Promega, Madison, WI). Using RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany), unincorporated ^{35}S-labeled UTP and DNA were removed and ^{35}S radioactivity concentration of the probes was adjusted by dilution to 500,000 counts/min/μL. The specific sequence of the two MMP-9 cDNA fragments was tested by DNA sequencing using a ABI 310 automatic sequencer, which confirmed the base pair sequences corresponding to 1,139 to 1,599 and 1,751 to 2,326 bp, respectively, of the MMP-9 cDNA (accession no. J05070).

In situ *Hybridization*

A detailed description of the *in situ* hybridization has been reported previously (27, 32). In brief, under RNase-free conditions, 16 μL of a hybridization mixture of the ^{35}S-labeled RNA probe ($\sim 2 \times 10^6$ counts/min/slide) were added to 3 μm paraffin sections and incubated overnight at 55°C in a humidified chamber. The sections were washed twice in solutions of SSC [0.15 mol/L NaCl, 0.015 mol/L sodium citrate (pH 7.0)] containing 0.1% SDS and 10 mmol/L DTT and then washed 10 minutes for each step (2×, 0.5×, and 0.2 SSC) and incubated with RNase A [20 mg/mL in 0.5 mol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 7.2)] at 44°C for 10 minutes to remove nonspecifically bound riboprobe and subsequently washed in 0.2× SSC as above. Sections were dehydrated in ethanol solution containing 300 mmol/L ammonium acetate, soaked in an autoradiographic emulsion (Ilford Imaging UK Ltd., Mobberley, United Kingdom), and exposed for 7 days at 4°C after which the sections were developed. Finally, the sections were counterstained with Mayer's H&E, dehydrated with ethanol, and mounted.

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