In Vitro Determination of Basement Membrane Invasion Predicts Liver Metastases in Human Gastrointestinal Carcinoma

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ABSTRACT

We described previously (H. Imamura, et al., Cancer Res., 54: 3620-3624, 1994) a quantitative and reproducible 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for tumor cell invasiveness that uses a water-repellent, paraffin-treated Chemotaxicell chamber to produce a uniform Matrigel layer. In the present experiments, we studied 71 human gastrointestinal carcinomas, including 53 maintained as xenografts in nude mice and 18 fresh surgical specimens. We found a correlation between metastatic behavior and the percent invasion (PI) calculated from the MTT assay. Tumors producing liver metastases had a significantly higher PI than did tumors without liver metastases (P < 0.01), and seven of nine fresh tumors with a PI greater than 1.0 showed liver metastases within 2 years. No significant correlations were noted between the PI and clinicopathological factors. In the tumor xenografts, type IV collagenase activity was significantly higher in tumors with clinically evident liver metastases than in those without liver metastases (P < 0.05). Colorectal carcinomas with liver metastases and a high PI showed higher expression of matrix metalloproteinase 9 than matrix metalloproteinase 2 as assessed by gelatin zymography. Thus, the invasion-MTT assay is clinically useful for predicting liver metastases. Type IV collagenase plays an important role in the development of liver metastases from human gastrointestinal carcinoma.

INTRODUCTION

Liver metastasis is the major cause of morbidity and mortality in patients with gastrointestinal carcinomas. Although several therapeutic modalities, including surgery, chemotherapy, and radiotherapy, are available for the treatment of liver metastases, the survival rate of patients with a liver metastasis is still poor. If the development of liver metastases could be predicted following resection of the primary tumor, earlier treatment for liver micrometastases would be possible.

Tumor invasion and blood-borne metastases represent a complex, multistep process involving the destruction of basement membranes, which represent a barrier against tumor invasion (1, 2). Tumor cell interaction with the extracellular matrix, and in particular with the basement membrane, occurs at multiple stages in the metastatic cascade (3). Major components of basement membranes include type IV collagen, laminin, and heparan sulfate proteoglycan (4). Type IV collagenase attacks basement membrane collagen at a pepsin-resistant triple-helical domain to generate characteristic NH2-terminal and COOH-terminal fragments representing one-quarter and three-quarters of the protein, respectively (5). Both the 72-kDa type and the 92-kDa type IV collagenases are secreted as latent proenzymes; latency of the proenzyme is maintained by an interaction between an unpaired cysteinyl residue of the profragment and the zinc atom at the active site (6–8). Metastatic ability correlates positively with type IV collagenolytic activity in murine tumor models (9-12). Similar results have been observed with murine colon carcinoma cells (13) and c-Ha-ras transfected 3T3 cells (14). Secretion of latent 92-kDa type IV collagenase in gastrointestinal carcinomas correlates with the metastatic phenotype (15, 16).

In recent years an in vitro invasion assay using reconstituted basement membrane matrix (Matrigel) has been developed for investigating cancer cell invasion into basement membranes (17-21). In such systems, establishing a uniform horizontal layer of Matrigel in contact with the filter of the chamber is difficult because of the meniscus phenomenon. We have devised a MTT3 invasion assay that uses a uniform coating layer of Matrigel produced by a water-repellent treatment with paraffin, and a tetrazolium-based colorimetric MTT assay for counting invading cells (22). This in vitro assay is fully quantitative and reproducible. We have been applying it to a small number of tumor xenografts from human gastrointestinal carcinomas maintained in nude mice and have found more invasive ability in tumors with liver metastases than in tumors without them (23).

In the present study, we addressed the same question in a large number of human gastrointestinal carcinomas, including fresh surgical specimens, and explored tumor cell interactions with basement membrane components.

MATERIALS AND METHODS

Animals. Male athymic BALB/c nude mice (nu/nu) 6 to 10 weeks old (Clea Japan Inc., Tokyo) were used in this study. The animals were kept in a laminar-flow rack under specific pathogen-free conditions throughout the experiments.

Tumors. Seventy-one human gastrointestinal carcinomas, including 53 tumor xenografts in nude mice (8 esophageal carcinomas, 9 gastric carcinomas, 21 colorectal carcinomas, 5 carcinomas of the bile duct, and 10 pancreatic carcinomas), and 18 tumors obtained directly at surgery (13 colorectal carcinomas, 4 carcinomas of the bile duct, and 1 gastric carcinoma) were used in this study. The passage numbers of the tumor xenografts ranged from 3 to 15. When a xenograft had grown to approximately 2 cm in diameter, it was resected under aseptic conditions and minced with scissors in a Petri dish containing RPMI 1640 (Nissui Co., Tokyo), supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml) purchased from Life Technologies, Inc. (Grand Island, NY). Fresh tumor specimens, about 1 cm in diameter, were processed directly for this study. Full-thickness tumors were sampled from the serosal aspect. Within 30 min of resection, they were placed in RPMI 1640 with antibiotics for 1 h and then minced.

Cell suspensions were harvested after a 30-min treatment with an enzyme cocktail including 0.2 mg/ml DNase (Sigma, St. Louis, MO), 0.5 mg/ml Pronase (Calbiochem, La Jolla, CA), and 0.2 mg/ml of collagenase IV (Worthington Biochemical, Freehold, NJ) in Hanks’ solution (Nissui Co.). Cancer cells were harvested with over 90% viability by using a mono-poly resolving medium (M-RPM, ICN Biomedicals Ltd., Oxfordshire, United Kingdom) and were adjusted to a density of 2 × 106 cells/100 µl with RPMI 1640. All of the cell suspensions were used immediately after preparation.

Invasion-MTT Assay. The invasion-MTT assay was performed as described previously (22). Briefly, modified Chemotaxicell chambers (Kubota Co., Tokyo) treated with paraffin as a water repellent were used as the upper wells. Each filter was coated with 70 µl of a 50-fold dilution (0.2 mg/ml of

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3 The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMP, matrix metalloproteinase; PI, percent invasion; TIMP, tissue inhibitor of metalloproteinase.
Matrigel (Collaborative Research Co., Bedford, MA) for tumor cells to invade. After 72 h of incubation, cells that invaded the lower surface and penetrated the Matrigel-coated filter were counted by the MTT assay. A conditioned medium, obtained by incubating NIH3T3 cells for 24 h in serum-free medium (Azinomoto Co., Tokyo), was used as a chemoattractant. MTT (Sigma) was used in the colorimetric assay (24). DMSO (Nacalai Tesque, Kyoto, Japan) was used to dissolve the formazan crystals of MTT.

**Percent Invasion.** We defined the in vitro invasive ability of the tumor cells in the invasion-MTT assay as a PI. A standard curve was calculated from the absorbance of 1 × 10⁶ cells, and the PI was calculated using the formula:

\[
\text{PI} = \frac{\text{Absorbance of invaded cells}}{\text{Absorbance of seeded cells}} \times 100
\]

**Attachment Assay.** Matrigel was allowed to coat the wells in 24-well plates (Corning Costar Co., Oneonta, NY) at the same density as in the invasion-MTT assay, and was polymerized at 37°C. Cell suspensions were harvested from the human tumor xenografts and resuspended in RPMI 1640 supplemented with 0.1% FCS. The cells were seeded into the Matrigel-coated wells at the same density as in the invasion-MTT assay and were incubated at 37°C in a 5% CO₂ humidified atmosphere. After 5 h, unattached cells were removed by washing twice with FCS-free culture medium. Attached cells were counted by MTT assay, and the percent attachment was expressed as the number of adhering cells divided by the total number of cells in each well.

**Chemotaxis Assay.** The chemotaxis assay was carried out under the same conditions described for the invasion assay, except that 5 μg of type IV collagen (Life Technologies, Inc., Gaithersburg, MD) was used as a coating to permit cell attachment, as described previously (25). Cell suspensions harvested from human tumor xenografts were resuspended in RPMI 1640 supplemented with 0.1% FCS. The cells moved through the pores toward the attractant (conditioned medium from NIH3T3 Albino cells) and were counted by the MTT assay. Percent chemotaxis was calculated as the number of attracted cells divided by the total number of cells in each well.

**Type IV Collagenase Assay.** The measurement of type IV collagenase activity was performed using [3H]-acetyl anhydride-labeled type IV collagen purified from Engelbreth-Holm-Swarm sarcoma cells as a substrate. This assay was carried out essentially as described by Nakajima (10). Briefly, conditioned medium, activated by 0.5 mm 4-aminophenylmercuric acetate, was incubated with radioactive substrate (5000 cpm/tube) at 37°C for 6 h. Products that did not precipitate in 10% trichloroacetic acid and 0.5% tannic acid were measured by liquid scintillation counting, represented type IV collagen digests. Bacterial collagenase (Sigma) was used to make a calibration curve.

**Gelatin Zymography.** To identify the types of collagenolytic enzymes in the colorectal carcinomas that had a high PI, gelatin zymography was performed in SDS gels using 7.5% polyacrylamide separating gels copolymerized with 0.1% Triton X-100 in 7.5% acrylamide gel. Gels were rinsed with 2.5% (v/v) Triton X-100 in 50 mM Tris-HCl buffer (pH 7.5). Gels were stained with 0.05% (w/v) Coomassie brilliant blue G-250 in a mixture of 10% isopropanol and 10% acetic acid and then destained in the same solution without dye. Gelatinolytic enzymes were detected as transparent bands on the background of Coomassie blue-stained gelatin. Prestained standard molecular weight marker proteins (Bio-Rad, Beverly, MA) were used as references.

**Statistical Analysis.** The Mann-Whitney U test, Kruskal-Wallis test, and Spearman rank correlation test were used for statistical analysis; P < 0.05 was considered to indicate statistical significance.

**RESULTS**

**PI of Xenograft Tumors and Liver Metastases.** During more than 4 years of follow-up, liver metastases have occurred in 22 of the 53 patients from whom the human gastrointestinal carcinoma xenografts were derived (including 8 with both liver and pulmonary metastases, 12 with both liver and i.p. dissemination, and 4 with both liver and bone metastases). Among the other 31 cases, there were 11 cases of i.p. dissemination, 7 local recurrences, 4 lymph node metastases, and 1 pulmonary metastasis. The PI values of tumors with liver metastases were significantly higher than those of tumors without liver metastases (Fig. 1A). However, no correlation was found between extrahepatic metastatic behavior and the PI. Of the 22 liver metastases, the 6 tumors with metachronous liver metastases had PIs similar to the 16 tumors with synchronous liver metastases (data not shown). The original sites of the 53 tumor xenografts included 23 primary lesions, 12 liver metastatic sites, 9 lymph nodes, 7 disseminations, and 2 local recurrences. No correlation was found between these sites and the PI (data not shown). However, PI values were higher when the primary lesions produced liver metastases than when they did not (Fig. 1B).

**PI of Fresh Tumors and Liver Metastases.** To eliminate the possibility that a high PI was associated with liver metastases only in tumor xenografts, we examined the correlation between the PI and clinical metastatic behavior using fresh tumors (Fig. 2). Recurrences, including metastases, were assessed after 2 years of follow-up. The PIs of the nine patients’ tumors without liver metastases were less than 1.0. Four of those patients have survived without liver metastases for more than 2 years. However, seven of nine patients with a PI greater than 1.0 developed liver metastases within 2 years, including two patients who were free of liver metastasis based on ultrasonography at the time of surgery but who developed liver metastases within 12 months.

**Type IV Collagenase Activity, Percent Adhesion, or Percent Chemotaxis, and Liver Metastases.** To study the basis of the correlation between the PI and clinical liver metastases, type IV collagenase assays, adhesion assays, and chemotaxis assays were performed. The type IV collagenase activity of tumors with clinical liver metastases was significantly higher than that of tumors without clinical liver metastases (Fig. 2). Metastases with synchronous liver metastases had PIs similar to the 16 tumors with synchronous liver metastases (data not shown). The original sites of the 53 tumor xenografts included 23 primary lesions, 12 liver metastatic sites, 9 lymph nodes, 7 disseminations, and 2 local recurrences. No correlation was found between these sites and the PI (data not shown). However, PI values were higher when the primary lesions produced liver metastases than when they did not (Fig. 1B).
metastases was significantly higher than that of tumors without metastases. No significant difference was seen between these two groups in percent adhesion or percent chemotaxis (Table 1). These results suggest that the high PI of tumors with liver metastases mainly reflects high type IV collagenase activity.

Collagenolytic Enzyme in Colorectal Carcinoma. Zymographic analysis was performed in tumor lines of colorectal carcinomas with a high PI to identify the types of collagenolytic enzymes involved. (In the tumor lines of 21 colorectal carcinomas, 8 primary tumors were examined.) The same tumor lines were simultaneously analyzed with the invasion-MTT assay. Zymographic analysis revealed a good correlation between the secretion of MMP-9 (92 kDa collagenase/gelatinase) into serum-free conditioned medium and the presence of liver metastases (Fig. 3).

Human colorectal carcinomas derived from the primary lesions with metastachronous (Fig. 3A, Lanes 2 and 3) or synchronous liver metastases (Fig. 3A, Lanes 1 and 4) had higher expression of MMP-9 than tumors without liver metastases (Fig. 3B, Lanes 1–4). MMP-9 expression correlates with a high PI in colorectal carcinoma, providing a basis for the high PI in primary tumors showing or developing liver metastases.

DISCUSSION

We examined and compared the PI for various sites of involvement including lymph node, peritoneum, and bone metastases, as well as local recurrences. The PIs of specimens from various sites were significantly higher when liver metastases were present. The PIs of specimens derived from primary lesions with liver metastases also were significantly higher than those of primary lesions without liver metastases (Fig. 1). This suggests that an ability to invade the basement membrane is required for blood-borne liver metastases.

Fidler and Hart (27) originally viewed metastasis as a highly selective competition favoring the survival of a subpopulation of tumor cells preexisting within a heterogeneous primary tumor. The term "dynamic heterogeneity" indicated the development of a dynamic equilibrium in which the frequency of metastatic variants in a given clonal population would be controlled by the rates of generation and loss of the variants (28, 29). In opposition to this concept, Kerbel et al. (30) recently proposed a "growth dominant theory" postulating that the metastatic subpopulation dominates the primary tumor mass early in its growth. Our results may support the "growth dominant theory" because we could predict the presence of liver metastases by assessment of the PI in tumors derived from the primary lesion. Moreover, measurement of the average level of a molecular marker in a primary tumor sample is likely to reflect the general metastatic tendency of the entire tumor (31).

The invasion-MTT assay measures the ability of the cells to attach to the matrix, degrade the matrix, and migrate toward a chemotaxant. These events are considered to be important steps in the metastatic spread of tumor cells through basement membrane in vivo (17, 18). To define the mechanism linking clinical liver metastases to the PI as assessed by the invasion-MTT assay, we investigated these abilities using adhesion assay, type IV collagenase assay, and chemotaxis assay. Adhesion and chemotaxis assays were performed under the same conditions as the invasion-MTT assay. We measured type IV collagenase activity because many investigators have indicated that...
MMP enzymes, especially type IV collagenase, are central to tumor-cell-mediated proteolysis of the extracellular matrix (2, 9, 10, 31–33). We investigated the relationship between the PI and MMP-2 and MMP-9 using gelatin zymography because these MMPs can degrade basement membrane components and are essential for tumor invasion (10, 31, 33). The results demonstrated that cells from tumors producing liver metastases showed a high PI, mainly because of high type IV collagenase activity. In addition, zymographic analysis demonstrated that MMP-9 positively influenced the PI value in colorectal carcinoma (Fig. 3). MMP-2 had no association with PI values in our study. Several studies have shown a correlation between metastatic potential and type IV collagenase activity (10, 25, 34, 35) or expression of type IV collagenase mRNA (36). Immunological studies using antibodies against MMP-9 have reported staining at invasive tumor margins in colorectal carcinoma (15). Moreover, a distinct pattern of MMP-9 mRNA expression has been seen in human colorectal carcinoma and liver metastasis by in situ hybridization (16). Direct evidence of the role of MMP-9 in tumor metastasis was shown by studies using transfection with a MMP-9 expression vector (37) and a hammerhead ribozyme that inhibits the expression of MMP-9 (38). Our results support these studies and prove a correlation between the PI and the development of liver metastases.

Jeziorska et al. (15) observed that MMP-9 in colorectal carcinomas overexpressed in a higher proportion of advanced stage tumors (Dukes C and D) by immunohistochemical staining. Murnane et al. (39) demonstrated that MMP-2 was not as highly expressed as MMP-9 in colorectal carcinomas, and its level did not correlate significantly with the Dukes stage by zymographic analysis. Our data indicate that MMP-9 expression may correlate with liver metastases as well as with high PI values of colorectal carcinomas.

The role of stromal cells, particularly macrophages, in the production of MMP-9 in human colorectal cancer specimens has been studied previously (15, 36). In our gelatin zymography, stromal cells were excluded from cell suspensions by using a mono-poly resolving medium under approximately 20% (data not shown). Our results suggest that the presence of stromal components of tumor may influence the MMP level. Additional studies are needed to define the colorectal carcinoma-stromal cell reactions involved in the regulation of MMPs and TIMP productions.

MMP activity in vivo is thought to be regulated, in part, by natural tissue inhibitor proteins, such as TIMPs (15, 31, 33). We performed zymographic analysis not on protein isolated from the specimens but on conditioned media from which the specimens were derived because the analysis of conditioned media allowed us to assess type IV collagenase under the same in vitro conditions as did the invasion-MTT assay. The PI was assessed in vitro by the invasion-MTT assay; therefore, endogenous inhibitors were presumably not present in the conditioned media from which these human carcinoma samples were derived. Thus, the invasion-MTT assay that was used to define the mechanism linking clinical liver metastases to the PI did not examine the role of TIMPs.

In conclusion, we developed the invasion-MTT assay (22) and investigated a correlation between PI and liver metastasis in human gastrointestinal carcinomas. The PI values of tumor xenografts that were derived from human gastrointestinal carcinomas had a highly significant correlation with liver metastasis in the patients from whom the tumors were derived. These findings were corroborated using cells taken directly from fresh tumor specimens at the time of surgery. In this study, we verified that the invasion-MTT assay is a valid in vitro assay that has a clinical correlation. Furthermore, this assay has the possibility of being developed as a predictive assay.

The invasive potential of tumor cells, defined as PI by the invasion-MTT assay, is affected mainly by type IV collagenase activity and especially by MMP-9 in colorectal carcinomas. Additional studies are required to define the molecular mechanism of metalloprotease secretion and activation.

REFERENCES


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