Inhibition of Gelatinolytic Activity in Tumor Tissues by Synthetic Matrix Metalloproteinase Inhibitor: Application of Film in Situ Zymography

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ABSTRACT

Inhibition of gelatinolytic activity in implanted tumor tissues by oral administration of N-biphenyl sulfonfonyl-phenylalanine hydroxamic acid (BPHA), a selective matrix metalloproteinase (MMP) inhibitor, was demonstrated by means of film in situ zymography (FIZ). Active-MMP-2 but not pro-MMP-2 showed gelatinolytic activity in FIZ, whereas both forms of MMP-2 were found to be active in conventional zymography. A mixture of either tissue inhibitors of metalloproteinase-2 or BPHA with active-MMP-2 resulted in inhibition of gelatinolytic activity in FIZ but not in zymography. Thus, FIZ, but not zymography, could detect net MMP activity in tumor tissues. When a specimen from Ma44 human lung cancer xenograft was subjected to FIZ, gelatinolytic activity was markedly detected with precise localization in the tumor tissues. The gelatinolytic activity detected in Ma44 tumor tissues was found to be mainly derived from MMPs because the gelatin-degrading activity was inhibited by pretreatment of the tumor specimen with MMP inhibitors. Oral administration of BPHA but not (−)BPHA, an enantiomer of BPHA lacking MMP inhibitory activity, successfully inhibited the MMP activity localized in Ma44 tumor tissues in both a dose-dependent and time-dependent manner. The data presented in this report showed for the first time that oral administration of synthetic MMP inhibitor could inhibit the net activity of MMP activity in tumor tissues, suggesting the usefulness of the FIZ technique for determining the effective dose of MMP inhibitor in clinical studies.

INTRODUCTION

MMPs have been considered to be essential for the invasion and metastasis of tumor cells and for angiogenesis (1–8), and to be potential target molecules for a new class of antitumor agents (9, 10). A number of low molecular weight synthetic MMP inhibitors are under development, and some are presently being investigated in clinical trials (11–16). One of the problems for clinical trials is the lack of an appropriate biological marker for determining the effective dose. Unlike cytotoxic anticancer agents, the MMP inhibitors have no limiting factors of adverse events, such as toxicity against blood cells or the gut. Therefore, as a surrogate maker, the detection of inhibition of MMP activity locally or systemically has been considered (17).

MMP activity is mainly regulated by two mechanisms. First, the secreted latent proenzyme forms of MMPs must undergo proteolytic activation. Second, ubiquitous TIMPs can interfere with MMP proteolytic activation and enzymatic activity. The presently available antibodies do not distinguish between proenzyme and proteolytically processed forms of MMPs. Colocalized TIMPs can prevent MMP activity even if the enzymes are in active form. Thus, immunohistochemistry does not provide information regarding the functional state of these enzymes. Biochemical studies of tissue extracts preclude localization of cell type exhibiting activity and association of TIMPs or inhibitors with enzymes, and they raise the possibility of artificial activation of the enzymes attributable to tissue disruption. Thus, conventional biochemical techniques such as zymography are expected to have limited availability for assessing the net functional activity of MMPs in tissues.

A promising new technique is a recently described in situ zymography, which can identify gelatinolytic activity in normal and pathological tissues, including cancer (18–22). This technique allows preservation of tissue structure with gelatinolytic activity, but has limitations regarding reproducibility and quantitation.

We have developed this technique by preparing cross-linked gelatin film having uniform thickness and properties. By controlling the degree of cross-linking, we were able to obtain a gelatin membrane suitable for localization of MMP activity in cancer tissues. We applied this FIZ technique to evaluate the biological activity of our recently developed MMP inhibitor, BPHA. Reported here, for the first time, is direct evidence for inhibition of gelatinolytic activity at tumor tissues by oral administration of synthetic MMP inhibitor.

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2 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; FIZ, film in situ zymography; BPHA, N-biphenyl sulfonfonyl-phenylalanine hydroxamic acid; p-ABSF, 4-(2-aminoethyl) benzenesulfonyl fluoride HCl.
MATERIALS AND METHODS

Animals and Tumors. Athymic BALB/c nude mice (female, 7–9 weeks old) were purchased from CLEA Japan Inc. (Tokyo, Japan). Ma44 human lung squamous cell carcinoma was provided by Dr. T. Komiya (Kinki University Medical School, Osaka, Japan) and was maintained by in vitro passage using Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS (Life Technologies, Inc., Rockville, MD).

Chemicals. BPHA and (−)BPHA were synthesized in Shionogi Research Laboratories (23, 24). 1,10-Phenanthroline was purchased from Sigma (St. Louis, MO). p-ABSF was purchased from Fuji Pure Chemical Industries, Ltd. (Osaka, Japan).

Enzymes and Inhibitor. Active human MMP-2 and its proenzyme were purchased from Oncogene Research Products (Cambridge, MA). Human recombinant TIMP-2 was purchased from Fuji Chemical Industries (Toyama, Japan).

Gelatin Zymography. Gelatin zymography was carried out as described elsewhere (25). Briefly, enzyme was applied to nonreduced SDS-PAGE using a 7.5% gel containing 0.1% gelatin. After electrophoresis, the gel was soaked in 2.5% Triton X-100 solution at room temperature with gentle shaking for 1 h. The gels were then incubated overnight in reaction buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM CaCl₂, 0.01% Brij-35] at 37°C and stained with Coomassie Brilliant Blue.

Specimens. Nude mice were inoculated either s.c. or i.p. with 5 × 10⁵ or 1 × 10⁵ of Ma44 cells, respectively. Two weeks after tumor inoculation, test compounds suspended in vehicle (saline including 0.4% Tween 80, 0.5% carboxy-methylcellulose, and 0.9% benzylalcohol) were p.o.-administered. At 1–24 h after treatment, the mice were sacrificed, the nodules were removed, and tumor specimens were obtained.

FIZ. Fresh tumor specimens (three cases) were dissected from tumor-bearing mice and embedded in cryomold O.C.T. 4583 Compound (Miles, Inc., Elkhart, IN) and immediately frozen on dry ice. These frozen blocks were then sliced sequentially using TISSUTEK II cryostat microtome (Miles, Inc., Elkhart, IN) and frozen on dry ice. These frozen blocks were then sliced sequentially using TISSUTEK II cryostat microtome (Miles, Inc., Elkhart, IN) and immediately frozen on dry ice. These frozen blocks were then sliced sequentially using TISSUTEK II cryostat microtome (Miles, Inc., Elkhart, IN) and indicated as numerical values.

RESULTS AND DISCUSSION

Gelatinolytic Activity of Active-MMP-2 by FIZ. The gelatinolytic activities of active- and pro-MMP-2 were examined by FIZ or zymography. The solution containing highly purified (>95%) human recombinant active-MMP-2 or pro-MMP-2 was serially diluted with buffer [50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 10 mM CaCl₂, 0.005% Brij-35], and an aliquot (1 μl) of the solution was spotted onto the gelatin-coated film. As shown in Fig. 1A, active-MMP-2 but not pro-MMP-2 showed gelatinolytic activity in a dose-dependent manner. When the enzymes were subjected to zymography, gelatinolytic bands appeared for both active- and pro-MMP-2 with corresponding molecular sizes (Fig. 1B). These results demonstrated that gelatinolytic activity derived from only active-MMP-2 was detected in FIZ without artificial activation of pro-MMP-2. Pro-MMP-2 used in these experiments appeared to be some active form in zymography (Fig. 1B, Lane 2), but this sample did not show any gelatin-digesting activity in FIZ (Fig. 1A, Lane 2). The analysis by means of matrix-assisted laser desorption ionization-time of flight/mass spectrometry demonstrated that the pro-MMP-2 sample contained 5–10% of TIMP-2 and <5%
Detection of Net Gelatinolytic Activity by FIZ.
Mixtures of various amounts of recombinant TIMP-2 and a fixed amount of active-MMP-2 were loaded on the gelatin-coated film. The gelatinolytic activity of active-MMP-2 was inhibited in the presence of TIMP-2 (Fig. 2A, Lane 5), whereas when the same mixture was subjected to zymography, a gelatinolytic band of active-MMP-2 was observed (Fig. 2C). The Mr 66,000 gelatinolytic band, i.e., intact active-MMP-2, indicates that MMP-2-TIMP-2 complexes were dissociated during separation in SDS-PAGE. Thus, these results demonstrated that FIZ but not zymography could detect net MMP activity.

Inhibition of Gelatinolytic Activity of MMP-2 by MMP Inhibitor by FIZ. Mixtures of various amounts of synthetic MMP inhibitors and a fixed amount of active-MMP-2 were loaded on the gelatin-coated film. As shown in Fig. 2A, the gelatinolytic activity of active-MMP-2 was completely inhibited by a broad MMP inhibitor, 1,10-phenanthroline (Fig. 2A, Lane 2), and a selective MMP inhibitor, BPHA (Fig. 2A, Lane 3), at 500 μM and 5 μM, respectively. In contrast, (−)-BPHA, an enantiomer of BPHA lacking MMP inhibitory activity, did not affect gelatinolytic activity (Fig. 2A, Lane 4). When the mixture was subjected to zymography, neither 1,10-phenanthroline nor BPHA exhibited their inhibitory activity (Fig. 2B). Therefore, FIZ but not zymography could be used to evaluate the local effect of MMP inhibitor.

Detection of Gelatinolytic Activity in Tumor Tissues by FIZ. Ma44 human lung cancer, which has been found to secrete MMP-2 predominantly in the culture supernatant (24), was implanted s.c. in the back of nude mice. The tumors with surrounding skin were subjected to FIZ (Fig. 3A) or standard H&E staining (Fig. 3B), as described in “Materials and Methods.” The gelatinolytic activity was markedly detected with precise localization in the tumor tissues. The region where gelatinolysis occurred was observed as white to pale blue in accordance with the tumor area, and other areas, such as skin with no gelatinolysis, were black. Thus, visualization of MMP activity could be achieved in situ using FIZ. The results from immunohistochemical staining using specific antibody against MMP-2 support the localization of MMP-2 in the tumor tissues (data not shown).

Inhibition of in Situ Gelatinolytic Activity by MMP Inhibitors. A similar degradation of gelatin substrate was observed in solid tumors adherent to the peritoneum or mesentery, which were formed in the mice i.p. inoculated with Ma44 tumor cells. The gelatinolytic activity at the tumor site was completely inhibited by dipping tumor tissues into HBSS containing 1 mM 1,10-phenanthroline, but not in the case of HBSS containing 1 mM p-ABSF, a selective serineprotease.
inhibitor, for 4 h before freezing (Fig. 4). These results indicate that the observed gelatinolytic activity was mainly derived from MMPs.

**Inhibition of in Situ MMP Activity by Oral Administration of BPHA.** Mice i.p.-inoculated with Ma44 tumor cells were p.o.-treated with the vehicle alone, 200 mg/kg of BPHA, or 200 mg/kg of (−)BPHA, and the tumor nodules were obtained at 2 h after administration. The cryosections were cut and subjected to FIZ to detect the remaining gelatinolytic activity in the tumor tissues. Oral administration of BPHA but not (−)BPHA resulted in potent inhibition of gelatin degradation in Ma44 tumors when compared with the vehicle control (Fig. 5). We then quantified the remaining gelatinolytic activity by image analysis. The numeral values indicating the brightness of the unit area of the whole slice from tumors treated with vehicle alone (Fig. 5A), 3 mg/kg of BPHA (Fig. 5B), 12.5 mg/kg (Fig. 5C), 50 mg/kg (Fig. 5D), and 200 mg/kg of BPHA (Fig. 5E) were 29.9, 24.1, 13.5, 10.9, and 3.8, respectively. Finally, analysis of the kinetics of inhibition of gelatinolysis by 200 mg/kg of BPHA showed that the inhibition of gelatin degradation in the tumor tissues reached a maximum at 1 h (Fig. 7B), and the effect of BPHA slowly decreased from 2 h onward (Fig. 7, C–E). The numeral values of the brightness of the unit area of tumor tissues showed the following order: vehicle > 3 mg/kg > 12.5 mg/kg > 50 mg/kg > 200 mg/kg of BPHA.

**Dose-dependent and Time-dependent Inhibition of in Situ MMP Activity in Tumor Tissues by Oral Administration of BPHA.** Ma44 tumor-bearing mice were p.o.-treated with 3, 12.5, 50, and 200 mg/kg of BPHA, and tumor nodules were obtained at 1 h after the administration. The cryosections were then subjected to FIZ. Although gelatinolysis was detected in the tumors from vehicle-treated mice (Fig. 6A), it was greatly reduced in the tumors from mice treated with 200 mg/kg of BPHA (Fig. 6E). Increasing doses of BPHA from 3 to 200 mg/kg resulted in more extensive inhibition of the gelatin degradation in the tumor tissues. The numeral values indicating the brightness of the unit area of the whole slice from tumors treated with vehicle alone (Fig. 6A), 3 mg/kg (Fig. 6B), 12.5 mg/kg (Fig. 6C), 50 mg/kg (Fig. 6D), and 200 mg/kg of BPHA (Fig. 6E) were 29.9, 24.1, 13.5, 10.9, and 3.8, respectively. Finally, analysis of the kinetics of inhibition of gelatinolysis by 200 mg/kg of BPHA showed that the inhibition of gelatin degradation in the tumor tissues reached a maximum at 1 h (Fig. 7B), and the effect of BPHA slowly decreased from 2 h onward (Fig. 7, C–E). The numeral values of the brightness of the unit area of tumor tissues showed the following order: vehicle > 3 mg/kg > 12.5 mg/kg > 50 mg/kg > 200 mg/kg of BPHA.
the whole slice from tumors at 0 h (Fig. 7A), 1 h (Fig. 7B), 2 h (Fig. 7C), 4 h (Fig. 7D), and 8 h (Fig. 7E) after administration of 200 mg/kg of BPHA were 30.8, 1.2, 3.1, 5.9, and 16.1, respectively. These results indicated that oral administration of BPHA significantly inhibited gelatin degradation in tumor tissue in both dose-dependent and time-dependent manner.

The in situ zymography technique has been previously reported to enable detection of the activity of MMPs in normal and pathological tissues (18–22). A more feasible and reliable technique should be FIZ because the quality of gelatin films is usually homogeneous among lots, and they can be stored for at least 1 year at room temperature. This study also shows the possibility of quantitative analysis of in situ degradation of gelatin. More precise and quantitative analysis between different...
films should be possible using internal standard of enzymatic activity, although validation and standardization of such an analysis remain to be required.

In this study, we demonstrated that FIZ could quantitatively detect the net MMP activity in terms of gelatinolytic activity in tumor tissues, and we offered direct evidence that oral administration of synthetic MMP inhibitor could markedly inhibit the net MMP activity in tumor tissues. A single dose of inhibitor was sufficient for local inhibition of gelatinolytic activity, which could be detected at as early as 1 h after administration and lasted until after 8 h. The inhibitory effect of BPHA could no longer be detected at 24 h after administration (data not shown). These results can provide important information for determining a practical dosing schedule in clinical studies.

Fig. 7 Time-dependent inhibition of in situ MMP activity in tumor tissues by oral administration of BPHA. Ma44 tumor nodules were obtained at 0 h (A), 1 h (B), 2 h (C), 4 h (D), or 8 h (E) after administration of 200 mg/kg of BPHA. Serial cryosections were subjected to FIZ (left panels) or H&E staining (right panels). Bar, 500 μm.

Acknowledgments

We thank F. Watanabe for providing the compounds and H. Tsuchiya for his valuable advice. We also thank H. Morita and N. Kikuchi for matrix-assisted laser desorption ionization-time of flight/mass spectrometry analysis.

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