A Quantitative Evaluation of Active Gelatinolytic Sites in Uterine Endometrioid Adenocarcinoma Using Film in situ Zymography: Association of Stronger Gelatinolysis with Myometrial Invasion

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Collagen matrix degradation by malignant tumor cells plays an essential role in the process of tumor invasion and metastasis. The purpose of this study was to detect in situ gelatinase activity in endometrioid adenocarcinomas of the uterine corpus. In order to carry out quantitative evaluation, autoexposure time (AET) on gelatin-coated film (film in situ zymography: FIZ) was measured. The gelatinase activity was located primarily within cancers and was prominently suppressed by the addition of a chelating agent to the film. This suggests that matrix metalloproteinases (MMPs) play an important role in the gelatinase activity. The gelatinase activity in the normal endometrium is almost negligible, despite positive immunoreactivity for MMP-2 and -9. Tumor tissues that had invaded more than half of the myometrium showed significantly higher activity than those that had invaded less than half. There was no significant difference in gelatinase activity among tumor stages, grades, vessel invasion or immunoreactivity for MMPs, with the exception that stage 2b cancers showed higher activity than stage 1a. The study suggested that the level of MMP-mediated gelatinolysis is an important factor for myometrial invasion in uterine endometrioid adenocarcinoma. Thus, a quantitative assessment of active gelatinolysis using FIZ and AET should be a useful tool in evaluating in situ matriolytic activity in local myometrial invasion by uterine endometrioid adenocarcinoma.

Key words: Endometrioid adenocarcinoma — Myometrium — In situ zymography — Autoexposure time — Uterus


Extracellular matrix-degrading proteinases are thought to play an essential role in the processes of invasion and metastasis.1–3 Gelatinases, such as matrix metalloproteinases (MMPs)-2 and -9, are especially important in invasion and metastasis because of their ability to degrade type IV collagen, a major constituent of basement membrane.4, 5 Studies using gelatin zymography6–9 or in situ zymography10–13 have shown that local activation of latent zymogens or progelatinase correlates with invasion and metastasis in various cancers. Although it has been shown that mRNA for MMP-1, -2 and -9 is abundant in adenocarcinomas of the uterine corpus,14 the gelatinolytic activity and its location in these tumors have remained undetermined.

The purpose of this study was to clarify location and activity of in situ gelatinase in endometrial endometrioid adenocarcinomas by using film in situ zymography (FIZ). To carry out quantitative evaluation, we developed a simple and reproducible autoexposure time (AET) method.

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MATERIALS AND METHODS

Patients Endometrial endometrioid adenocarcinoma tissues were obtained from 31 patients who had given informed consent. The patients ranged in age from 30 to 83 years with an average age of 59. The clinicopathologic features of the patients are shown in Table I. Noncancerous tissues from 8 patients, 4 premenopausal and 4 postmenopausal, were used as control samples. The specimens were embedded and frozen in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo) and stored at −80°C until sectioning. Histology was assessed according to the criteria established by the WHO,15 and the stage and histological grading were those of the International Federation of Gynecology and Obstetrics (FIGO).16 Multiple serial sections of 4 μm thickness were obtained from each specimen to facilitate hematoxylin and eosin staining, in situ zymography, and immunohistochemical staining for MMPs. At least nine serial sections were used for FIZ in order to obtain the average AET of each individual case (see below).
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FIZ In situ gelatinase activity was detected, using two types of polyethylene terephthalate film. One, FIZ-GN, is a film coated with a 7 µm layer of crosslinked gelatin, but not bathed in a chelating solution. The other, FIZ-GI, is a film coated with a 7 µm layer of crosslinked gelatin, and bathed in a chelating solution. FIZ-GI films were used for the inhibition for MMPs. To make FIZ-GI films, FIZ-GN films were bathed in 1,10-phenanthroline solution (100 mmol/liter in 50% ethanol, adjusted to pH 7.4 with 0.1 mol/liter HCl) for 10 min, and dried at room temperature. FIZ-GN and FIZ-GI films were kind gifts from Fuji Photo Film Co., Ltd., Tokyo.

The serial frozen sections were mounted on these films. After incubation in a box with 100% humidity at 5°C or 37°C, films were allowed to dry at room temperature for 30 min, and were then stained with 1% Amido Black 10B (Wako Pure Chemical Industries, Ltd., Tokyo) for 15 min. After having been washed 3 times with distilled water, the films were first decolorized in 70% methanol with 10% acetic acid and 20% H₂O for 10 min and then dried. Amido Black 10B stains gelatin on film, leaving areas resolved by gelatinase unstained. The gelatinase activity is thus visible as a translucent or gray area, depending on the intensity of the activity.

AET For quantitative evaluation, the AET was measured by a Carl Zeiss Axioplan (Carl Zeiss Co., Ltd., Tokyo) focused on a designated area. When magnification and filament voltage were constant, AET depended on the field’s brightness, which reflected in situ gelatinolytic activity; the stronger the gelatinase activity, the lower the AET value. AET is a function of light source filament voltage, which should therefore be kept constant during experiments. The correlation between AET and filament voltage is shown in Fig. 1. The relationship between the two shows a curved line, and we adopted 6 volt as the most appropriate voltage. We used the average of the values of 9 serial sections, determined under a camera magnification of 5×2.5.

Experimental conditions for FIZ and AET In FIZ, regions where gelatinolytic activity is present appear as bright areas in a dark background; the bright areas correspond to sites where gelatin has been digested, and the dark areas to non-digested sites. Visible gelatinolysis occurred on FIZ-GN films at 37°C for 8 to 10 h. Gelatinolysis did not occur at 5°C even when the experiment was continued for 18 h. The extent of gelatinolysis was time-dependent. Fig. 2 shows the correlation between AET and incubation time in a representative case, with the incubation periods being 6, 10, 14, 18, and 22 h at 37°C. An incubation time of 14 h was selected for further studies. When the film was incubated longer than 22 h, both the tissue structures and the gelatin film were destroyed by gelatinolysis, making evaluation difficult.

Immunohistochemistry MMP-2/-9 immunoreactivity in the tissues was determined using frozen sections with monoclonal antibodies against MMP-2 and -9 with a dilution of 1:100 (Fuji Medicine Co., Ltd., Tokyo). The immunoreactivity was visualized by using freshly prepared

| Table I. Clinicopathologic Scores of Patients in This Study |
|-----------------------------|------------------|
| Clinicopathologic scores   | Number of cases  |
| Grade                       | Number of cases  | (%)   |
| 1                           | 20 (65)          |
| 2                           | 6 (19)           |
| 3                           | 5 (16)           |
| Stage                       |                  |
| 1a                          | 4 (13)           |
| 1b                          | 14 (45)          |
| 1c                          | 5 (16)           |
| 2a                          | 2 (6)            |
| 2b                          | 6 (19)           |
| Depth of myometrial invasion|                  |
| <1/2                        | 19 (61)          |
| ≥1/2                        | 12 (39)          |
| Vessel invasion             |                  |
| negative                    | 21 (68)          |
| positive                    | 10 (32)          |

Fig. 1. Relation between autoexposure time (AET) and filament voltage in a representative case.

Fig. 2. AET and incubation time in a representative case. AET becomes shorter as incubation time is longer.
diaminobenzidine (0.2 mg/ml) in Tris buffer that contained 0.005% hydrogen peroxide, counterstained with hematoxylin. A negative control was generated by replacement of the primary antibody with mouse IgG diluted to an equivalent concentration. The immunoreactivity was classified into two groups: the positive group, in which over 50% of cancer cells were positively stained; and the negative one, in which under 50% showed positive staining.

**Statistical analysis** The AET value from each case was expressed as mean ± standard deviation for comparison.
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Fig. 5. A. Autoexposure time (AET) and histological grades. B. AET and stages. C. AET of tumor tissues with invasion to the middle of the myometrium (<1/2) and those to the deeper half (≥1/2). D. AET and vessel invasion. E. AET and immunoreactivity for MMP-2 or MMP-9.

Fig. 6. Immunostaining of matrix metalloproteinase (MMP)-2. A, Localization of immunoreactivity for MMP-2 in cancer glands from the gelatinolytic area in Fig. 3B (original magnification ×400). B, Localization of immunoreactivity for MMP-2 in non-cancerous endometrial glands shown in Fig. 3D (original magnification ×400).
Statistical significance was determined by using Student’s t test, and P<0.05 was considered to be significant.

RESULTS

All cancer tissues showed gelatinase activity by FIZ to a certain extent (AET=0.164±0.144). The localization of gelatinase activity was heterogeneous within a given tumor and high activity was seen near the borders with normal tissues in some cancer tissues (Fig. 3, A and B). In contrast, the normal endometrium generally exhibited negligible activity (AET=0.86±0.145) (Fig. 3, C and D). The difference between normal and cancerous tissues was significant (P<0.001), but there was no significant difference between premenopausal and postmenopausal women (data not shown). Gelatinolytic activity was greatly diminished on the FIZ-GI film compared with the FIZ-GN film (Fig. 4), and the difference between FIZ-GN and FIZ-GI was significant (AET=0.164±0.144 in FIZ-GN vs. AET=0.439±0.324 in FIZ-GI, P<0.0001). Gelatinolytic activity did not differ among tumor grades: the AETs of tumor tissues of grades 1, 2 and 3 were 0.145±0.095, 0.094±0.092, and 0.325±0.245, respectively. No significant difference was found between any combination of two groups (Fig. 5A). AETs of stages 1a, 1b, 1c, 2a and 2b were 0.203±0.098, 0.179±0.182, 0.149±0.124, 0.295 (n=2, see Table I) and 0.073±0.042, respectively (Fig. 5B). The gelatinolytic activity of stage 1a tumors was significantly lower than that of stage 2b tumors (P<0.05) but no significant difference was observed in any other combination of two stages. The AET of tumor tissues with invasion up to the middle of the myometrium (AET=0.201±0.161) was significantly lower than that of those with invasion to the deeper half (AET=0.105±0.088, P<0.05) (Fig. 5C). There was no significant difference between the two groups with and without vessel invasion (Fig. 5D). Gelatinolytic activity in MMP-2/-9-negative cancer tissues (AET=0.67±0.12) was slightly lower than that in positive tissues (AET=0.32±0.09), but the difference was not significant (Fig. 5E).

In the neoplastic glands, gelatinase activity was found where MMP-2/-9 was positively immunostained (Fig. 6A). The gelatinolysis and immunoreactivity sites were almost identical. In contrast, control normal endometria showed negligible gelatinolysis, irrespective of occasional positive stainings for MMP-2/-9 in the glands (Fig. 6B).

DISCUSSION

FIZ has been used to assess in situ gelatinase activity in human ovarian neoplasms,12,17 brain tumors,10 nasal polyps,18 and breast cancers,13 but there have been few reports on in situ gelatinase activity of uterine endometrioid adenocarcinomas. Since there have been no reports on quantitative assessment by in situ zymography, we examined the localization and quantification of gelatinase activity in endometrioid adenocarcinoma by FIZ. We developed a simple and reproducible method for quantitative evaluation of gelatinolysis on FIZ using AET. AETs were successfully obtained with a microscope fitted with a camera equipped with an autoexposure device. Lower AETs indicate high gelatinase activity.

Our results show that, in cases of uterine endometrioid adenocarcinoma, gelatinase activity is significantly stronger than in the non-cancerous endometrium. When cancer tissues were applied to the chelating agent-containing FIZ-GI, the gelatinase activity was significantly but not completely suppressed. As the chelating agent greatly suppresses MMP activity by chelating metal ions, this finding suggests that MMPs play an important role in gelatinolysis.

It was also found that cancer tissues that invaded to the deeper half of the myometrium showed higher gelatinase activity than those that invaded only up to the shallower half. This indicates that gelatinase activity is closely related to the local invasion of tumor cells into the myometrium. These results agree to some extent with the finding by Iurlaro et al. that mRNA for MMP-2 and MMP-9 detected by in situ hybridization is significantly overexpressed depending on the degree of myometrial invasion and the grading from G1 to G3.19 Although the strength of in situ gelatinolytic activity did not correlate with grades, vessel invasion, or immunoreactivity for MMPs, a weak correlation was seen in stage; stage 2b cancers had higher activity than stage 1a (P<0.05). This finding also supports the notion that gelatinolytic activity associated with tumor cells is important in the local spread of endometrial cancer tissues.

The endometrium undergoes breakdown and regeneration during each menstrual cycle. Rodgers et al. report that MMPs are expressed in the endometrium in association with tissue breakdown and sloughing at the end of the menstrual cycle.20,21 In the present study, only negligible gelatinase activity was recognized in both pre- and postmenopausal normal control endometrial tissues. This is consistent with the absence of breakdown in the postmenopausal endometrium.

On the other hand, MMP-2/-9 was positively stained in immunohistochemical studies of the non-neoplastic endometrium. This agrees with the report of Maatta et al.,22 who showed by zymography that tumor tissues contain active MMP-2 and MMP-9, while normal endometria contain only proforms of MMPs. This may indicate the presence of an inhibitory substance in normal tissues, a substance not generally found in cancerous tissues. The activity of MMPs in the extracellular space is strictly controlled by MMP inhibitors, and tissue inhibitors for MMPs (TIMPs) have been reported to be abundant in reproduc-
tive tissues. Rodgers et al. were among the first to identify TIMP-1 mRNA in both endometrial epithelial and stromal cells with varying intensity of expression throughout the menstrual cycle. All these findings explain the virtual absence of gelatinolytic activity, irrespective of the presence of immunoreactive MMP proteins in non-neoplastic endometria. These results indicate that the presence of immunoreactivity for MMPs does not necessarily imply activity, and that FIZ is an appropriate and reliable method for determining the relevance of gelatinase activity in cancer.

We did not find any significant difference in the activity between MMP-positive and -negative cancer tissues, although it is possible to see a trend, in that positive can-
in cancer.

MMP-2/-9 may function in MMP-2/-9-negative cancer tissues. Second, MMPs in positive cancer tissues may be partially suppressed in a mechanism similar to, or different from, the suppression in non-cancerous endometrial glands. Finally, a smaller amount of MMP-2/-9-positive cells, i.e., under 50% of cells, is enough for the gelatinolytic activity. A combination of these explanations is also possible.

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