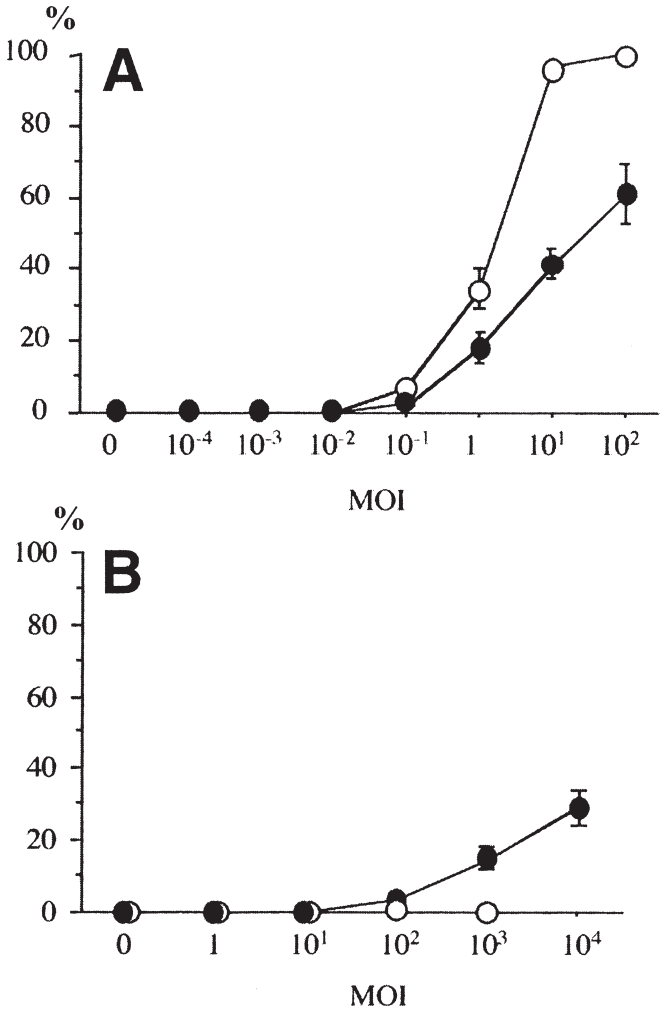

Fig. 51 Infectivity of Adex1CAlacZ (**A**) and AdAFPlacZ (**B**) in FU97 (●) and MKN28 (○) cells. β-galactosidase activity was determined by X-gal staining at 24 h later.

4) Enhancer/promoter-specificity of adenovirus vectors

To examine the enhancer/promoter-specificity of vectors, FU97 and MKN28 were infected with AdAFPlacZ, and β-galactosidase activity was determined 24 h later. X-gal staining revealed that β-galactosidase activity was evident in FU97 but not in MKN28 (Fig. 51B and Suppl. Fig. 3C). Since sensitivity to adenovirus vectors of MKN28 exceeded that of FU97, it is considered that the lacZ gene was driven by the AFP enhancer/promoter of the vector exclusively in the AFP-producing cell line. When the vector was infected at 10^4, 10^3, 10^2, and 10 MOI, 29.67±1.86%, 9.67±1.16%, 4.00±1.00%, and 1.33±0.33% of FU97 cells stained blue. The positivity was low in comparison with the findings when Adex1CAlacZ was infected, which suggested the difference in promoter activity between the AFP enhancer/promoter and the CAG promoter.

5) Cytotoxicity of AdAFPtk and GCV

FU97 was infected with AdAFPtk at 0, 10, 30, or 100 MOI, or exposed to 0, 1, 10, 10^2, 10^3, or 10^4 μM of GCV. When the viable cells were counted 8 days later, 100 MOI of AdAFPtk and over 100 μM of GCV had severe cytopathic effects on the cells, respectively (Fig. 52). Thus, we used AdAFPtk at 0.3, 3, 10, and 30 MOI, and GCV at 0, 1, and 10 μM, in the following experiments.

6) Effects of the suicide gene therapy *in vitro*

Combined use of AdAFPtk (3, 10, and 30 MOI) and GCV (1 and 10 μM) significantly reduced the number of viable FU97 cells in comparison with findings when AdAFPlacZ and GCV were combined (Fig. 53). These results suggest that transduction of the HSVtk gene increased the cytotoxicity of GCV in the AFP-producing cell line. In addition, the effects depended on the dose of either AdAFPtk or GCV. Contrarily, no similar effect was evident regarding MKN28, even when AdAFPtk at 30 MOI and 10 μM of GCV were used in combination (Fig. 54).

7) Summary and perspectives

Gastric adenocarcinoma producing AFP is one of the poor-prognostic neoplasms. In searches for new therapeutic strategies against AFP-producing gastric cancer, we examined the efficacy of suicide gene therapy which has been effective on AFP-producing hepatoma. The HSVtk gene was transduced into an AFP-producing gastric adenocarcinoma cell line, FU97, using adenovirus vectors carrying the constructed AFP enhancer/promoter element, followed by GCV administration. Expression of the transgene was evident in FU97 but not in an AFP-nonproducing gastric adenocarcinoma cell line, MKN28, which meant that AFP

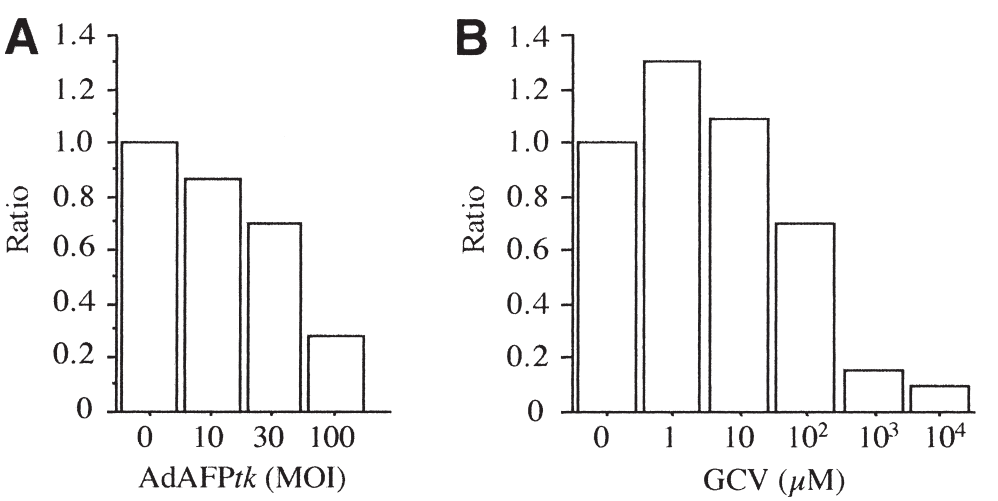

Fig. 52 Cytopathic effects of AdAFPtk (**A**) and GCV (**B**) to FU97. Eight days later, viable cells were counted and each ratio to that of cultured FU97 without AdAFPtk or GCV were calculated.

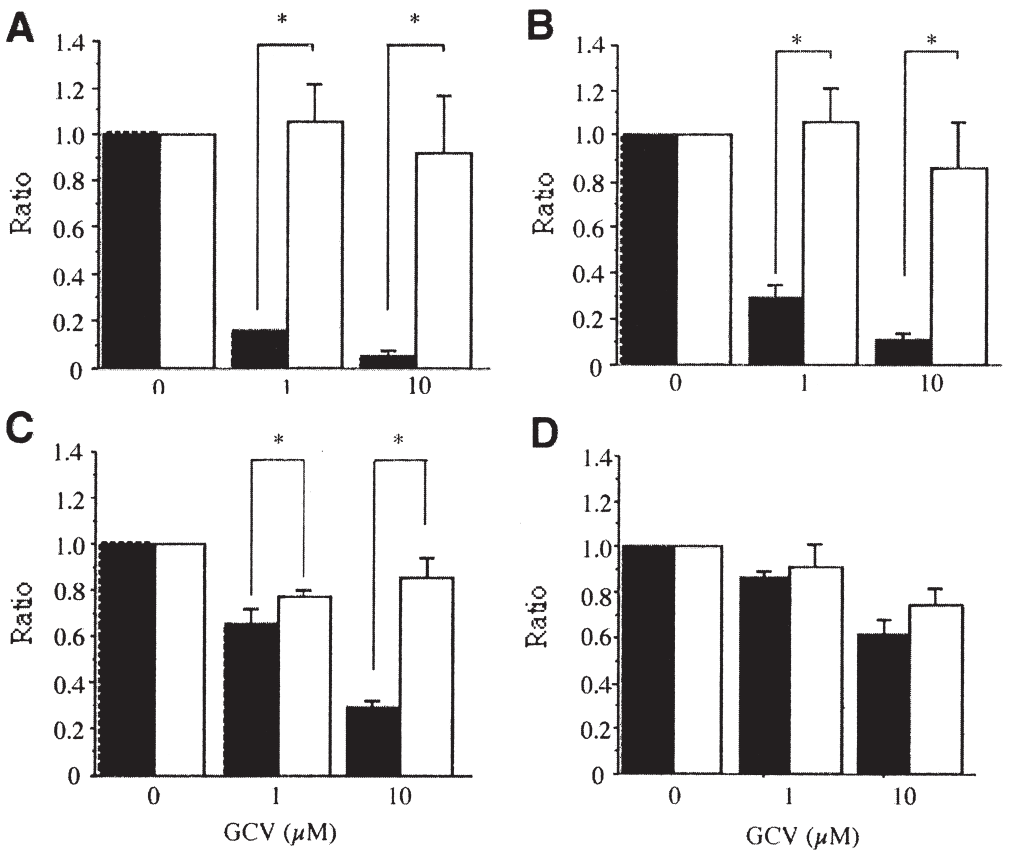

Fig. 53 Effects of suicide gene therapy. FU97 was infected with AdAFPtk (closed columns) or control AdAFPlacZ (open columns) at MOI of 30 (**A**), 10 (**B**), 3 (**C**) or 0.3 (**D**) followed by GCV administration. At day 7 after addition of GCV, viable cells were counted and each ratio to that of AdAFPtk infected FU97 without GCV were calculated. *: $p<0.05$.

enhancer/promoter-specific transcriptional targeting was achieved by the vectors. Viability of FU97 but not of MKN28 significantly decreased after the suicide gene therapy *in vitro*.

Therapeutic application of the AFP enhancer/promoter-specific transfer of the *HSVtk* gene followed by GCV administration against AFP-producing gastric cancer deserves attention and further research.

Role of Matrix Metalloproteinases and Related Tissue Inhibitors in Cancer Tissues

1. Clarification of the active gelatinolytic sites in human ovarian neoplasms using *in situ* zymography (120)

1) Introduction

Matrix metalloproteinases (MMPs) are zinc endopeptidases required for degra-

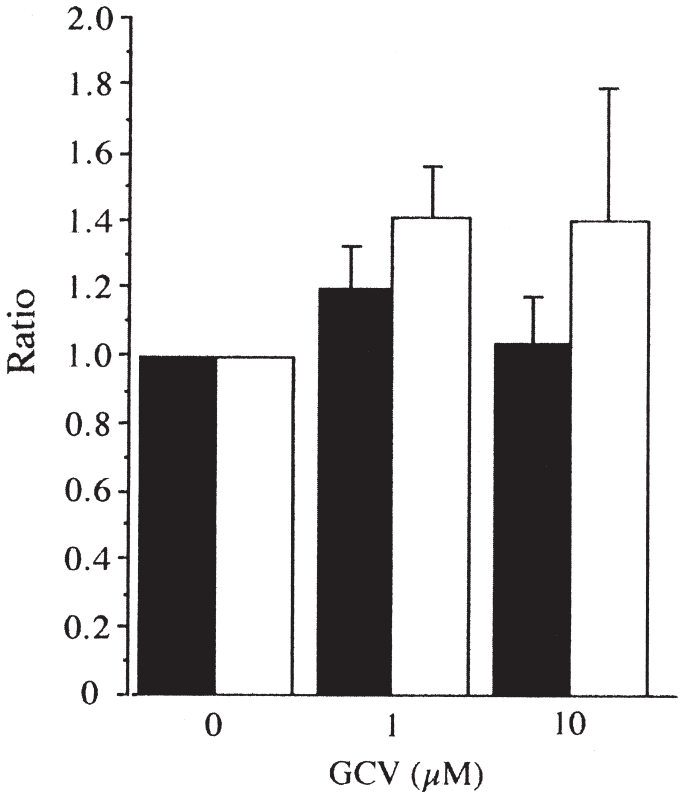

Fig. 54 No effect of the suicide gene therapy using AdAFPtk and GCV in MKN28, a gastric adencarcinoma cell line without AFP producution.

dation of the extracellular matrix in tumor growth and invasion (121). Expression of these enzymes in human malignant neoplasms has been studied immunohistochemically (IH), by *in situ* hybridization (ISH) and using zymography (ZG) (122-124). The matriolytic activity *in vivo* exerted by MMPs is determined by multifarious interactions among proteolytic activators and specific inhibitors such as tissue inhibitors of MMPs (TIMPs) (125-127). Neither IH, ISH, or ZG pinpoints the localization of matriolytic activity *in vivo*; IH detects MMPs by their antigenicity, with both the proenzymatic and activated MMPs, as well as both the TIMP-bound and -unbound forms, being stained equivalently. Similarly, ISH detects the location of mRNA expression, but not the activity itself, and ZG detects matriolytic activity of MMPs both in TIMP-bound and -unbound forms when gel electrophoresis is used. The methodological properties listed above limit our understanding of the role of matriolytic activity *in vivo*, since they do not identify cellular localization of true matriolytic activity. The newly developed *in situ* zymography (ISZ), using a gelatin film over unfixed frozen tissues, addresses this issue by detecting in situ gelatinolytic activity on tissue sections (128, 129). ISZ detects the sum of the following *in vivo* activities not saturated by TIMPs; a weak gelatinolytic activity of the proenzymatic MMPs, a strong activity of the activated MMPs, and

gelatinolytic activities of enzymes other than MMPs. We used ISZ to determine the *in situ* localization of gelatinolytic activity in ovarian tumors and the adjacent tumor stroma. Different grades or histologic subtypes of ovarian tumors may have different *in situ* gelatinolytic activities, as has been suggested by IH, ISH, and ZG (122–124). Thus, our important hypothesis as regards *in situ* gelatinolytic activity is that the location of activity may be variable, depending on histologic grades and/or subtypes.

2) *In situ* zymography (ISZ)

ISZ detects the *in situ* gelatinolytic activity as areas unstained by Amido Black 10B. Treatment of the films with 100 mM of 1,10-phenanthroline suppressed the unstained areas in all adenomas and borderline tumors, but it did not completely suppress them in most of the adenocarcinomas. The unstained areas were dose-dependently inhibited in preincubation of tumor tissues with CGS27023A (130); in Case 4 (mucinous adenocarcinoma) and in Case 13 (clear cell adenocarcinoma), gelatinolysis was considerably blocked at 50 μM of CGS27023A, and the activity was almost completely inhibited at 100 μM. Thus, the activity was attributed mainly to MMPs.

The correlation between significant gelatinolysis and histological subtypes is shown in Table 7. In normal ovaries, corpus luteum cells showed obvious gelatinolysis (Fig. 55). Capillary endothelial cells and granulosa cells expressed weak gelatinolysis. Primary oocytes and corpus albicans showed no gelatinolysis. In four benign tumors, no complete or obvious gelatinolysis was seen, but in one mucinous adenoma, a weak gelatinolysis was seen in the tumor cytoplasm.

Malignant neoplasms showed various gelatinolytic activities. In two clear cell and four mucinous adenocarcinomas, the complete to obvious gelatinolytic activity had pattern A (Fig. 56A and B), which was not seen in those of serous adenocarcinomas or borderline tumors, although a scattered weak activity was seen in the tumor cytoplasm of serous adenocarcinomas (5/6). In four mucinous and three serous adenocarcinomas and one mucinous and one serous borderline tumors,

Table 7 Significant gelatinolysis in tissue specimens detected by *in situ* zymography.

Histology (no. of cases)	Cellular and Tissue Localization of Gelatinolysis			
	Tumor Cytoplasm	Tumor-Stromal Junction	Tumor Stroma	Cystic Fluid
Mucinous Ca (6)	4/6	1/6	0/6	4/6
Serous Ca (6)	0/6	1/6	5/6	3/6
Clear cell Ca (6)	2/6	0/6	6/6	0/6
Mucinous borderline tumor (2)	0/2	1/2	0/2	1/2
Serous borderline tumor (2)	0/2	0/2	1/2	1/2
Mucinous adenoma (3)	0/3	0/3	0/3	0/3
Serous adenoma (1)	0/1	0/1	0/1	0/1

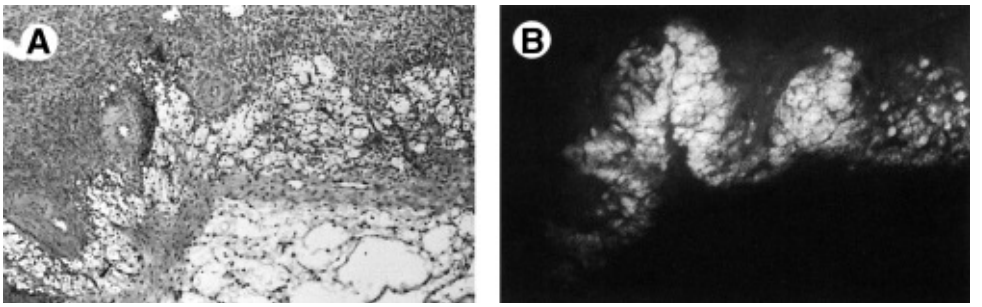

Fig. 55 *In situ* gelatinolytic activity in a corpus luteum. Granulosa lutein cells (**A**: HE staining) showed gelatinolysis (**B**: *in situ* zymography).

pattern D was seen, and was usually stronger than that in the cytoplasm of tumor cells lining the cysts. One mucinous and one serous adenocarcinoma and a mucinous borderline tumor had gelatinolytic activity of pattern B (Fig. 56C and D). Histologic examination revealed mucin prolapse into the stroma at the tumor-stromal junction and silver staining showed a partially disrupted basement membrane. Most serous and all clear cell adenocarcinomas expressed strong gelatinolysis and had pattern C, especially where tumor cells were dispersed in the desmoplastic stroma (Fig. 56E and F).

3) Immunohistochemistry (IH)

IH showed that MMP-2, MMP-9 and MMP-7 were stained in the epithelial cells and/or stroma of carcinomas and borderline tumors (Table 8). MMP-9 was also positive in some capillary endothelial cells. In mucinous carcinomas and borderline tumors, MMP-7 was positive in the epithelium (4/5). In one mucinous adenocarcinoma, MMP-9 was stained both in the epithelium and junctional stroma. Most mucinous carcinomas and borderline tumors showed weak and scattered positivity for MMP-2 and/or MMP-9 in the epithelium. Two of three serous adenocarcinomas stained for MMP-2 and MMP-9 both in tumor cells and desmoplastic stroma where tumor cells were diffuse, whereas papillary tumor nests with a scant stroma showed weak or no staining. In two of five clear cell adenocarcinomas, MMP-9 and MMP-7 were positive in the tumor cytoplasms, especially where tumor cells were dispersed. MMP-2 was positive in the hyalinized stroma in one clear cell adenocarcinoma. Normal ovary and mucinous adenomas showed no significant staining, except for staining for MMP-7 in the cytoplasm and for MMP-2 in the stroma in one mucinous adenoma.

4) Summary and perspective

Tissues from 26 human ovarian common epithelial tumors were examined to determine where and how gelatinolytic activity was present, in relation to tumor-stromal interaction and histologic types. For this purpose, we made use of *in situ* zymography, a newly developed technique using gelatin-coated film. Gelatinolytic

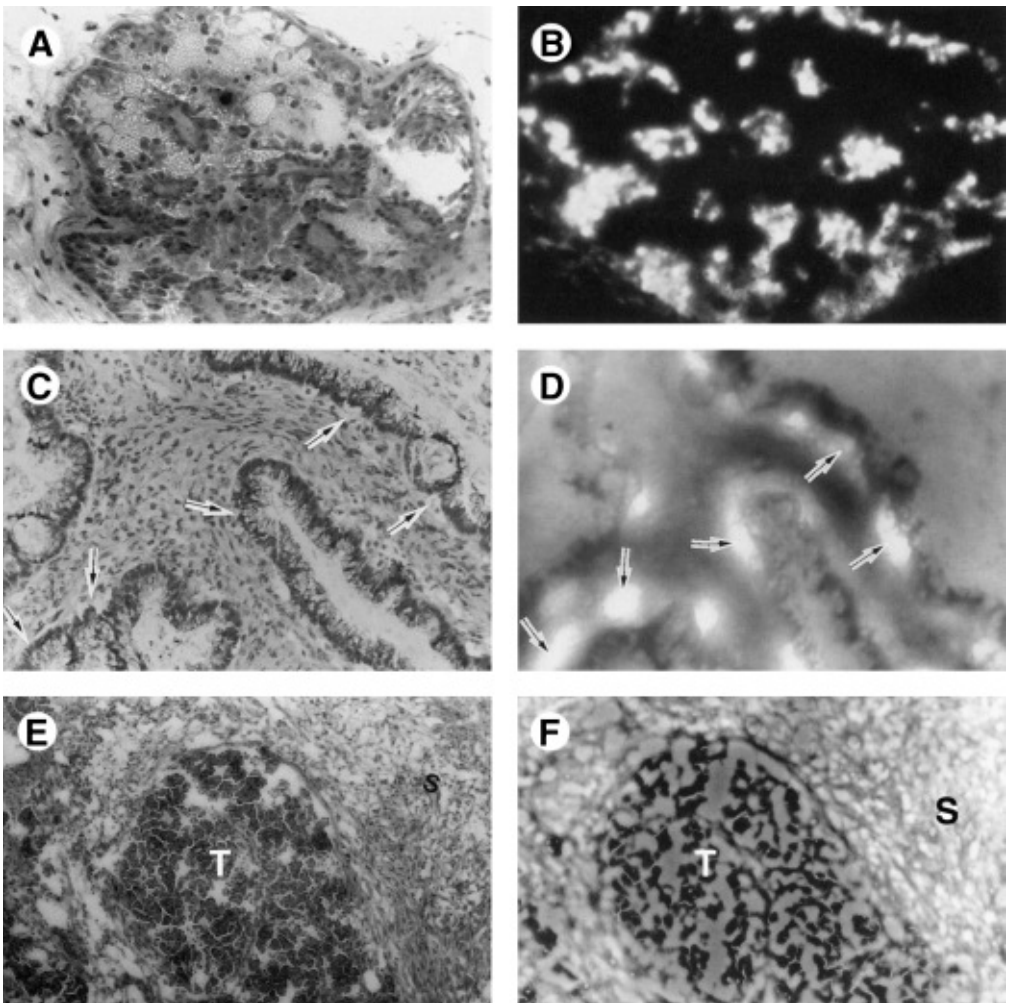

Fig. 56 Different gelatinolytic patterns shown with *in situ* zymography. Three different patterns, cytopathic pattern (**A** and **B**), tumor-stromal junction pattern (**C** and **D**, arrows indicate junction) and stromal pattern (**E** and **F**, S indicates stroma and T indicates tumor), were observed. **A**, **C** and **E** are HE staining and **B**, **D** and **F** are *in situ* zymography.

Table 8 Summary of immunohistochemical location of MMPs in ovarian tumors.

	MMP-2		MMP-7		MMP-9	
Histology (no. of cases)	Tumor Cytoplasm	Stroma	Tumor Cytoplasm	Stroma	Tumor Cytoplasm	Stroma
Mucinous Ca (4)	1/4	0/4	3/4	0/4	1/4	1/4
Serous Ca (3)	2/3	2/3	2/3	0/3	2/3	2/3
Clear cell Ca (5)	1/5	1/5	2/5	0/5	2/5	0/5
Mucinous borderline tumor (1)	0/1	1/1	1/1	0/1	0/1	0/1
Serous borderline tumor (2)	1/2	1/2	1/2	0/2	1/2	0/2
Mucinous adenoma (2)	0/2	1/2	1/2	0/2	0/2	0/2
Normal ovary (1)	0/1	0/1	0/1	0/1	0/1	0/1

activity was evident in ovarian carcinomas and in borderline tumors. Benign tumors had no or only weak activity. Four tissue localization patterns of gelatinolysis were identified: pattern A) tumor cytoplasm, pattern B) tumor-stromal junction, pattern C) stroma, and pattern D) cystic fluid. Mucinous cystadenocarcinomas showed A and/or D patterns. One mucinous and one serous adenocarcinoma and one mucinous borderline tumor had a B pattern. Most serous and all clear cell adenocarcinomas showed strong gelatinolysis of C pattern, especially in the desmoplastic stroma, an area where the tumor cells were dispersed. Immunohistochemically in 12 adenocarcinomas and 3 borderline tumors, the tumor cytoplasm was positive for MMP-2 (5 cases), MMP-7 (9 cases) and MMP-9 (6 cases). Stromal components were positive for MMP-2 in 5 and for MMP-9 in 3 cases, but not for MMP-7. MMP antigens were mostly distributed in an almost identical pattern with that seen with *in situ* zymography. *In situ* zymography clarified the cellular localization of active gelatinolysis in human ovarian neoplasms, findings that support the view that interaction between tumor and stroma is critical for tumor growth. This newly developed method contributes to a better understanding of biological features of ovarian malignancies.

2. Expression of matrix metalloproteinases and related tissue inhibitors in the cyst fluids of ovarian mucinous neoplasms (131)

1) Introduction

MMPs are zinc endopeptidases required for degradation of the extracellular matrix during normal embryogenesis and in tissue remodeling (132) and they also play an important role in tumor growth, invasion and metastasis (121). MMPs are classified according to substrate specificity, comprising collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11) and others (133, 134). Studies on the expression of MMPs in human tumors have led to new insights into biology of the lesions. At least focal disruptions of basement membranes of epithelial ovarian tumor cells, as evidenced by histology and immunohistochemistry for basement membrane materials, have been shown, and more importantly, the disruptions correlated with the expression of MMP-2 by tumor cells (135). As the lysis of the basement membrane matrix is an initial step of cancer invasion and metastasis, the expression of matrix proteinases by tumor cells has an obvious importance in ovarian cancer biology. Expression of these enzymes in ovarian cancers has also been shown in other studies (136, 137). Although the role of proteolysis has been studied also in ovarian carcinoma cell lines and in ascites (138, 139), the relationship between species or activities of tumor-derived MMPs and biologic aggressiveness of ovarian tumors has remained largely unclear. Proteinases derived from tumor cells and secreted to the stroma play an important role in the establishment of gross patterns of ovarian epithelial neoplasms and such proteinases may be detected in cystic fluids without being intervened by normal tissue-derived proteinases. Therefore, cystic fluids appear to be an appropriate

source in analyzing specificity and interrelationship of tumor-derived enzymes with proteolytic or anti-proteolytic activity. In addition, proteolytic activities in cystic fluids have obvious implications, per se, in the event of rupture and dissemination of cystic lesions. The aim of the present study, therefore, is to analyze species, interrelationship and clinical significance of these enzymes in ovarian mucinous tumors.

2) Detection of gelatinolytic activity in cystic contents and cytoplasm of mucinous tumor cells by *in situ* zymography

In situ zymography clearly demonstrated gelatinolytic activity within cytoplasm of neoplastic epithelial cells (Suppl. Fig. 4). Matriolytic activity of mucinous contents was even more intense. Malignant tumor cells exhibited a stronger activity than benign ones did. There were scattered, weak activity in the tumor stroma and the activity was not so intense as neoplastic cells and mucinous fluids. The gelatinolytic activity was inhibited dose-dependently with a preincubation of tumor tissues with CGS27023A. Gelatinolytic activity was blocked in more than half of the tumor cells at 50 μM of CGS27023A and the activity was almost completely inhibited at 100 μM.

3) Metalloproteinase activity in mucinous cystic fluids by zymography

The enzymatic activity in mucinous ovarian cystic fluids was examined using gelatin and casein zymography. In gelatin zymography, 40 times dilution was required to obtain unequivocal bands in all carcinoma/borderline fluids and in most adenoma fluids, whereas in the fluids from 4 of the adenomas, 20 times dilutions was sufficient. In casein zymography, all carcinomas/borderline fluids required 10 times dilution, and adenoma fluids required 2–5 times dilution. Gelatin zymography was primarily concerned with two gelatinolytic enzymes with approximate molecular masses of 92 and 72 kDa, which correspond to MMP-9 and MMP-2, respectively. In gelatin zymography, the major concerns were trypsin and MMP-7 at 25/23 (140) and 29 kDa, respectively. Western blotting confirmed that the activity at these molecular weights had antigenic determinants for MMP-9 (92 kDa), MMP-2 (72 kDa), MMP-7 (29 kDa), and trypsin (25 and 23 kDa).

In all carcinomas and borderline fluids, both MMP-9 and MMP-2 activities were clearly detected (Fig. 57). The MMP-9 band was also detactable in 12 of 15 adenoma fluids. When analyzed by densitograph, the pattern of these gelatinolytic activities differed depending on degree of malignity. In carcinoma/borderline fluids, both proenzymatic and activated MMP-9 (124) were detected. In one mucinous carcinoma, the MMP-9 band was completely shifted toward the size for the activated form, and no proenzymatic form was detected. Activated MMP-9 was detecyted in 7 of 15 adenoma fluids, although its presence was weak when compared to the findings in carcinoma/borderline category. The frequency and clarity of activated MMP-9 was the highest in the carcinoma category, followed by

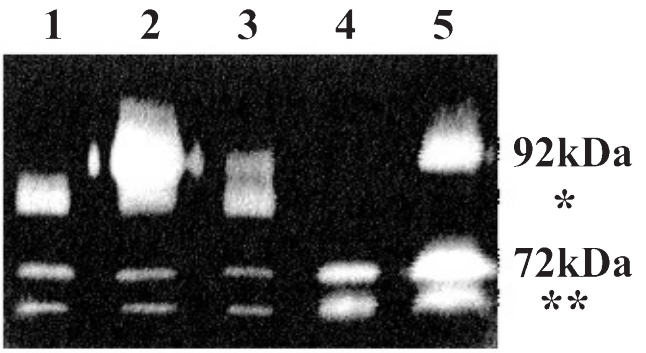

Fig. 57 Detection of MMP activity by gelatin zymography in the cyst fluids of ovarian mucinous tumors. 92 kDa is MMP-9 and 72 kDa is MMP-2. *: activated MMP-9, **: activated MMP-2. Lanes 1 and 2 are mucinous adenocarcinomas, lane 3 is mucinous borderline tumor, and lanes 4 and 5 are mucinous cystadenomas.

the borderline category. MMP-2 activity was almost ubiquitously present throughout the cystic lesions, including the control functional cysts. Activated MMP-2 was frequently evident and could appear in any histological category.

Trypsin and MMP-7 were detected in cystic fluids by casein zymography (Fig. 58). Both positive rates of trypsin and MMP-7 presence in carcinoma and borderline fluids were higher than that of adenoma but not significant, statistically (Table 9). On the other hand, MMP-3 was detected in all carcinomas and borderline fluids and the positive rate was statistically significant compared with that of adenoma ($p<0.01$). In the control group, there was no evidence of the production of any these caseinolytic enzymes.

4) Quantification of MMPs and TIMPs in mucinous cystic fluid by ELISA

The observed concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 were shown in Table 10. The concentration of MMP-9 was highest in mucinous car-

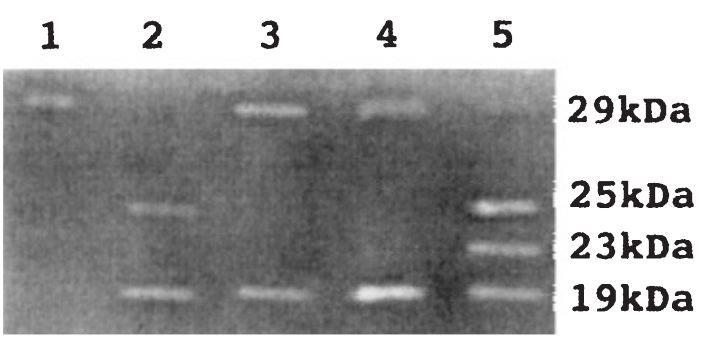

Fig. 58 Detection of MMP-7 and trypsin by casein zymography in the cyst fluids of ovarian mucinous tumors. MMP-7 is detected at 29 kDa and its activated form is 19 kDa. 25 and 23 kDa are trypsin type 2 and type 1, respectively. Lanes 1 to 4 are mucinous cystadenomas and lane 5 is mucinous borderline tumor.

Table 9 Summary of the positive rates of MMP-3, MMP-7 and Trypsin in mucinous cystic fluids.

Histologic category (number)	MMP-3 positive rate(%)	MMP-7 positive rate(%)	Trypsin positive rate(%)
Carcinoma (8)	100	62.5	37.5
Borderline tumor (3)	100	66.7	33.3
Adenoma (15)	53.3	40.0	13.3
Control cyst (7)	0	0	0

Table 10 Summary of TIMP and MMP concentrations in mucinous cystic fluids (ng/ml).

Histologic category (number)	MMP-9 (range)	MMP-2 (range)	TIMP-1 (range)	TIMP-2 (range)
Carcinoma (8)	1968 ± 2706[a] (300−6000)	1411 ± 1697 (200−4300)	17023 ± 18213[c] (5500−52000)	183 ± 225 (8−640)
Borderline tumor (3)	926 ± 460 (480−1400)	499 ± 166 (384−690)	22183 ± 8982[e] (16100−32500)	70.7 ± 47.4[f] (18−110)
denoma (15)	423 ± 720[b] (0−2600)	544 ± 352 (35−1250)	6563 ± 5163[d] (1800−19000)	343 ± 234[g] (15−760)
Control cyst (7)	0.264 ± 0.452 (0−0.95)	1005 ± 1113 (350−3500)	2777 ± 933 (1550−4150)	186 ± 151 (15−760)

$p<0.05$ in *a* vs *b*, *c* vs *d*, *e* vs *d*, and *f* vs *g*.

cinomas, followed by borderline tumors. TIMP-1 was highest in borderline tumors and was also higher in carcinoma than it was in adenoma fluids. Both MMP-9 and TIMP-1 concentrations were statistically higher in carcinoma/borderline fluids than in adenoma fluids ($p<0.05$). Mucinous carcinomas were the highest level of MMP-2 contents, although no significant difference was seen among the categories. TIMP-2 was less prevalent in carcinoma/borderline fluids than in adenoma fluids.

The molar ratios of TIMP-1/MMP-9 and TIMP-2/MMP-2 were shown in Table 11. For TIMP-1/MMP-9, this ratio was lowest in mucinous carcinomas, followed by borderline tunors. The TIMP-2/MMP-2 ratio was also lower in carcinoma/borderline tumors than in adenomas.

5) Summary and perspectives

Expansion of ovarian cystic neoplasms often involves invasion to and destruction of extracellular matrix. We examined species, interrelationship and clinical significance of MMPs and TIMPs in neoplastic cysts of ovarian mucinous tumors,

Table 11 Summary of TIMP/MMP molar ratios in mucinous cystic fluids.

Histologic category (number)	TIMP-1/MMP-9 ratio (range)	TIMP-2/MMP-2 ratio (range)
Carcinoma (5)	55.2 ± 27.2(27.5−91.5)	1.21 ± 1.21[a](0.04−3.09)
Borderline tumor (3)	104.1 ± 96.2(40.7−214.8)	0.46 ± 0.27[b](0.16−0.68)
Adenoma (15)	1670.0 ± 3414.7(4.3−12689.7)	2.54 ± 1.50[c](0.52−5.77)
Control cyst (7)	9591.5 ± 603.4(0−10018.1)	0.90 ± 0.64(0.52−1.05)

$p<0.05$ in *a* vs *c*, $p<0.01$ in *b* vs *c*.

using zymography (*in situ* zymography, gelatin zymography and casein zymography), enzyme-linked immunosorbent assay and western blotting. Matriolytic activity was demonstrated within cytoplasm of mucinous epithelial lining cells by *in situ* zymography, attributing the origin of intracystic matriolytic activity mainly to these cells. The concentration of MMP-9 was statistically higher in mucinous carcinomas ($p<0.05$) than in benign and borderline ones. TIMP-1, which combines with MMP-9, was also higher ($p<0.05$) in malignancies than in benign ones. The ratios of MMP-9/MMP-2 and the occurrence of activated forms of MMP-9 and MMP-2 correlated with the degree of malignancy, whereas the molar ratios of TIMP-1/MMP-9 and TIMP-2/MMP-2 were higher in benign ones. Expressions of MMP-3 or trypsin in the fluids were frequently accompanied by activation of MMP-7 and MMP-9. These observations verified the usefulness of ovarian cystic fluids in the analysis of matriolytic activity of ovarian cystic neoplasms. In addition, they support the concept that the presence and interactions of tumor-derived enzymes with matriolytic and antimatriolytic activity are modulators of growth pattern and biologic aggressiveness of cystic ovarian tumors.

3. Expression of matrix metalloproteinase in the fluids of renal cystic lesions (141)

1) Introduction

Recent progress in radiological imaging system increased clinical approaches to cope with asymptomatic renal lesions including benign cysts and renal cell carcinomas. Available modalities include contrast media, rapid sequence CT and MR imaging. Radiological approaches alone do not always diagnosis of cystic renal lesions (142). According to the Bosniak classification (143), a certain extent of malignant cystic disease is included in categories II and III (143, 144), and in such cases surgical approaches may be required to determine adequate treatment. Though cytology and serum tumor markers have also been studied, their value in making a diagnosis is limited to cases of renal malignancy (145, 146).

Matrix metalloproteinases (MMPs) are zinc endopeptidases required to degrade the extracellular matrix (132) in embryogenesis and tissue remodeling as well as in tumor progression (121). MMPs are classified according to substrate specificity. MMP categories include collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11) and others (121). Some species of MMPs have been reported to play an important role in renal malignancies (147–149). Although analyses of proteolytic molecules in blood plasma or homogenized tumor tissues can explain in part the tumor progression, they are modified by nonneoplastic fibroblasts, inflammatory cells and plasma derived MMPs (148, 150). Recent studies showed that proteinases derived from tumor cells can be detected in fluid of cystic lesions (120, 131), therefore cyst fluid can be used to analyze matriolytic activity of the local lesions.

As hypothesized that matriolytic enzymes might be expressed in renal cyst fluid

and would specifically reflect biological activities in accordance with malignancy. We investigated the expression of MMPs in the cyst fluids of the kidney, using gelatin zymography and enzyme linked immunosorbent assay.

2) Determination of metalloproteinases in cyst fluid of the human kidney

In gelatin zymography, two gelatinolytic enzymes of an approximate molecular mass of 92kDa and 72kDa, corresponding to MMP-9 and MMP-2 respectively, were the major concerns (Fig. 59). Western blotting confirmed that the proteolytic bands were immunoreactive, and the patterns were identical. In gelatin zymography, 20–50 times dilution was required to obtain unequivocal bands in all carcinoma fluids, whereas in most benign cysts, 1–2 times dilution was sufficient. Zymography was repeated for each case when the protein concentration was adjusted to 1–5 mg/dl until expressions of MMPs were confirmed. The MMP-2 band was detected ubiquitously in all but two case of cystic renal disease and these proved to be benign cysts of acquired cystic disease of the kidney (ACDK). MMP-9 was detected in 7 of 8 (87.5%) cystic renal cell carcinomas while 12 of 14 benign cysts were negative for MMP-9 ($p < 0.01$) (Table 12). The activated forms (131) of MMP-9 (88 kDa) and MMP-2 (68 kDa) were not detected in any specimen (Fig. 59).

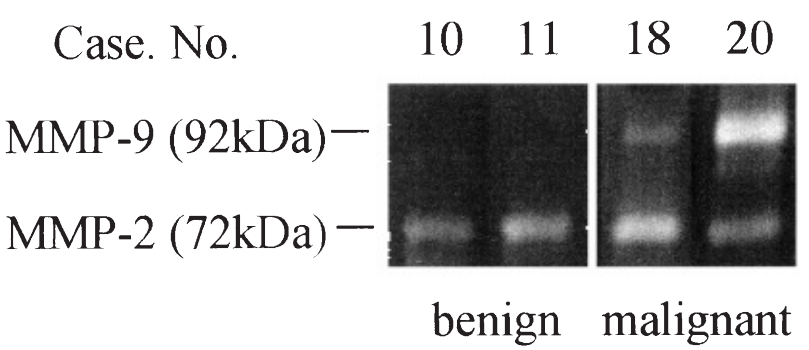

Fig. 59 Detection of MMP expression in cystic fluids of kidney diseases with cystic formation. Case no. 10 and 11 are benign simple cysts, and case no. 18 and 20 are cystic renal cell carcinomas.

Table 12 Positive rate of MMP-2 and 9 in renal cystic fluids determined on gelatin zymography.

Clinicopathological Category	No. Cases	No. Matrix Metalloproteinase (%)	
		2	9
Benign cyst, including simple cysts +acquired cystic disease of kidney	14	12 (85.7)	2 (14.3)
Renal cell Ca, clear cell histology	8	8 (100)	7 (87.5), $p < 0.01$ vs. Cyst

3) Concentration of MMP-2 and MMP-9 in renal cyst fluid determined by ELISA

The concentrations of MMP-2 and MMP-9 were statistically higher in cystic renal cell carcinomas than in benign lesions ($p<0.01$, $p<0.01$, respectively) (Fig. 60). In two benign cysts associated with ACDK, in which the MMP-2 band was not seen in gelatin zymography, the concentration of MMP-2 was also undetectable. MMP-2 concentrations in all but one benign cyst were lower than 100 ng/ml, while in 7 of 8 malignant cysts they were higher than 100 ng/ml. MMP-9 concentrations in malignant cyst fluids were between 1.0 and 400 ng/ml. All three Bosniak-IV cases showed over 10 ng/ml, while most benign cysts were below detectable levels. The highest concentration in benign cases was 7 ng/ml (Bosniak-I).

4) Summary and perspectives

Cystic lesions of the kidney are common conditions usually diagnosed according to imaging studies. Although simple cysts are easy to diagnose, the preoperative diagnosis of complicated cystic lesion can be difficult. There has been little information on the biological activity of cyst fluid and the association with clinicopathological findings. We analyzed the expression of MMPs in cyst fluids of benign and malignant renal cystic lesions in an attempt to clarify matriolytic activity in the cyst. Twenty-two cyst fluids from renal cystic lesions (14 benign cysts and 8 cystic renal cell carcinomas) were included in this study. The presence of MMP-2 and MMP-9 in fluids was examined using gelatin zymography and ELISA. Expression of MMP-2 was ubiquitously observed zymographically except for two benign cysts associated with ACDK. MMP-9 was detected in 7 of 8 carcinomas, but only in 2 of 14 benign cysts ($p<0.01$). Concentrations of both MMP-2 and MMP-9 were significantly higher in cystic carcinomas than in benign cysts ($p<0.01$, $p<0.01$, respectively).

Our data support the notion that matriolytic enzymes such as MMP-2 and

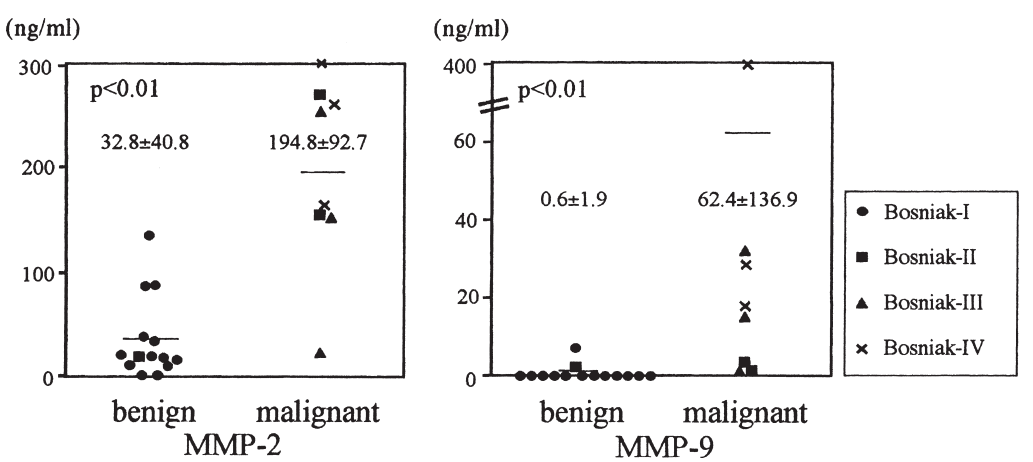

Fig. 60 Correlation of MMP-2 and 9 in renal cystic fluids on ELISA. Bars indicated mean value.

MMP-9 are expressed in cyst fluid of renal cystic diseases. There is a significant difference of MMPs concentrations between benign cysts and cystic renal cell carcinomas. Presence of these enzymes into cyst fluid may reflect aggressiveness of cystic renal cell carcinoma. These observations contribute to a better understanding of biological behavior in human renal cystic changes.

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