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行政院國家科學委員會補助專題研究計畫成果報告

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※ Extracellular Matrix-degrading Proteinase expression ※
※ in human ovarian carcinoma ※
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計畫主持人：童寶玲

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□赴國外出差或研習心得報告一份

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Extracellular matrix-degrading proteinase expression in human ovarian carcinoma

Introduction:

Ovarian carcinoma is one of the leading causes of death in Taiwan and the number of cases has been increasing. However, metastasis has occurred in the majority of patients by the time of diagnosis. During tumor progression, the invasive capacity of the malignant cells to penetrate tissue barriers for further metastases has been reported to be related to the presence of extracellular matrix-degrading proteinases, also called as matrix metalloproteinases (MMPs) (1).

Two important enzyme of the MMP family, MMP-2 (gelatinase A, 72-kDa type IV collagenase) that degrade gelatins and basement membrane collagen types IV and V, are of particular interest in ovarian cancer. Overexpression has been reported to be associated with increased invasive and metastatic potential (2,3), though its correlation with the progression of ovarian cancer is still controversial in some reports (4). MMP activity, in vivo, is regulated in part by its natural inhibitor, TIMP-2 (tissue inhibitors of metalloproteinases) (5). Elevated proteinase secretion unchecked by an equivalent rise in inhibitor levels can enable a tumor cell to invade neighboring tissues and metastasize. The balance of MMP-2 to TIMP-2 expression has been reported as an essential factor in the aggressiveness of cancer (6). More recently, a membrane-type MMP (MT-MMP-1) with a transmembrane domain has been cloned and suggested to mediate cell surface activation of MMP-2 (7). The MT-MMP-1 might associate with TIMP-2, both acting together as a receptor for Pro-MMP2 and leading to the cleavage of zymogen. Strongin et al (8) proposed that MMP-2 activation may occur through a trimolecular complex containing MT-MMP-1, TIMP-2, and MMP-2. However, the relevance of such a model has not been demonstrated in vivo and the involvement of other components cannot be excluded.

Synthesis of MMP and TIMP has not been detected in the primary culture of normal ovarian surface epithelium (OSE) (9), but high levels of MMP-2 was detected in ovarian cancer cells derived from ascitic fluid, primary ovarian tumors and metastatic lesions (10).Localization of MMP-2 protein was found both in the stromal cells as well as the epithelail tumor cells (11). However, mRNA of MMP-2 and TIMP-2 were found primary localized in the stromal tissue of ovarian cancer (12,13). MMP2 was suggested to be secreted from the stromal cells and then bind to the ovarian cancer cell surface by Yung et al (14). On the other hand, MMP-2 protein was also reported to localize in the cytoplasm but not membrane in multiple cancer (15). The exact expression pattern of protein and mRNA of MMPs in the ovarian tumor cells need further studies in detail. Peritoneal mesothelial cells, which are involved in the peritoneal metastasis, were found to express high levels of MMP-2 and TIMP-2 (16). The interactions of cancer cells with the mesothelial cells are of particular interested, especially when MT-MMP-1 is considered.

Interactions between the cancer cells and mesothelial cells will be investigated as a co-culture system in this study and the gelatinolytic activities of both the pro-enzyme and the activated form of MMP-2 will be analysed by zymography.

Materials and Methods:

Tissues

Formalin-fixed, paraffin-embedded human ovarian tumor blocks from the surgical archives at National Taiwan University Hospital were retrieved and 4-μm serial

sections were taken. Histological evaluation including classification and grading of the malignant tumors were diagnosed by pathologists. The study include 49 tmors: 21 clear cell carcinoma, 12 serous papillary adenocarcinoma, 4 mucinous cystadenocarcinoma, 4 endometriod carcinoma and 8 krukenburg tumor.

Cell Culture

The human ovarian surgical specimens collected during operation were aseptically dissected into small pieces and placed in petri dish containing Dulbeecco's modified Eagle's medium (DMEM) solution with 15% heat inactivated fetal calf serum (FCS), 2 μM glutamine, and 100 μg/mL kanamycin and incubated at 37° C, 10% CO2 as stated (22). Passages of cells will be continued while confluence occurred. The newly established ovarian carcinoma cell line OVTW-59 was cultured in DMEM solution with 5% FCS.

Immunohistochemistry

Avidin-biotin (ABC) immunoperoxidase technique was used for Immunostaining for all human ovarian tumors as stated (23). The sections were deparaffined with xylene and dehydrated and then washed in PBSC (0.1 M phosphate buffer pH 7.4, 8.5% sucrose, 0.002% $CaCL_2$), incubated with 0.1% NaN_3 and 1% H_2O_2 to suppress endogenous peroxidase activity. Normal blocking solution (1:50 diluted) was added to remove any non-specific antibody bindings. Primary antibodies (MMP-2, Calbiochem-Novabiochem International, Cambridge, MA, clone 42-5D11, diluted at 1:20; and MMP-9 Calbiochem-Novabiochem International, Cambridge, MA, clone 56-2A4, diluted at 1:20) were added and incubated overnight at 4°C. After washing with PBSC, 1:200 diluted biotin labeled second antibody was reacted for 1 hour, washed and then reacted with ABC for 30 minutes at room temperature. 0.05% DAB (3,3'-diaminobenzidine-4 HCL in 0.05M Tris-HCL, pH 7.2) with 0.01% H_2O_2 was added for color development. Counterstaining using 0.01% OsO_4 was performed and the slides were mounted in 50% glycerol.

In Situ Hybridization

The distribution of mRNA encoding MMP-2 and TIMP-2 was determined in serially sections of human ovarian tumors, using anti-dig phosphatase. The following template cDNAs were used to generate antisense probes labeled with dig:

1. A 132 bp MMP-2 cDNA sequence corresponding to nucleotides 1517 to 1649 of human MMP-2, subcloned into TOPO vector (invitrogen) plamid, linearized with EcoRI and transcripted with SP6 and T7 RNA polymerase (Promega, Southampton UK).
2. A 303 bp TIMP-2 cDNA sequence corresponding to nucleotides 567 to 870 of human TIMP-2, subcloned as above described.

Both cDNA were obtained through RNA extraction of ovarian tumors, followed by reverse transcription and polymerase chain reactions. Using SP6 and T7 promoter, the RNA labeling kit (Boehringer Mannheim) was used to dig-label the sense and the antisense of MMP-2 and TIMP-2. dig-labeled riboprobes transcripted with T7 RNA polymerase were used ass negative controls.

In situ hybridization (ISH) of MMP-2 and TIMP-2 RNAs were performed as follow (24):

Serially cut 5 μm sections of all human ovarian tumors was deparaffined with xylene, rehydrated. 1 mg/ml of proteinase K was added at 37℃for 30 minutes, and then actylated at room temperature. The slides were washed in PBS (phosphate saline buffer 0.1M, NaH_2PO_4, o.1 M Na_2PO_4, 0.15 M NaCl), prehybridization solution (50% deionized formamide, 250 μg/ml salmon sperm DNA, 0.05% SDS, 1 x Denhart's solution, 4 x SSC, 50 mM sodium phosphate buffer, pH 6.5) and heated to 95 ℃for 5 minutes to denature the DNA in cells. The slides were chilled in ice and then hybridedwith 0.1 μg of dig labeled probe and incubated at 42 ℃ overnight. After hybridization, the slides were washed sequentially with 4xSSC, 2xSSc, 1xSSC and 0.5xSSC, and 10% blocking solution, followed by 1% blocking solution containing AP linked antibodies (1:500) was added and incubated at 4℃ overnight, followed by Maleic acid buffer and detection buffer. NBT and X-phosphate were added for color development and mounted.

In Vitro co-culture

Human mesothelial cell or fibroblast primary cultures were established from surgical specimen that are dissected andcultured in DMEM containing 10% FCS, 100 μg/ml kanamycin and incubated at 37 ℃, 10% CO_2. Primary cultures, with the first 8 passages of the ovarian tissues obtained from surgical specimen, at the concentration of $2x10^5$/ml was added to petri dishes. Equal amount or 2:1 ratio of mesothelial cells or firoblasts to ovarian primary culture cells were added to the petri dish for co-culture study.

Preparation of condition medium

The cells were cultured at $5x10^5$/ml in serum free DMEM overnight. Recombinant human transforming growth factor beta 1 (R & D systems, incorporation)at the concentration of 0.5ng/ml, 1 ng/ml, 2 ng/ml and 5ng/ml were added to the culture medium for 24 hours before analysis of gelatinase activities. Alternatively, 0.5ng/ml or 1 ng/ml TIMP-2/MMP2 complex, obtained form human rheumatoid synovial fibroblasts (Calbiochem) were added to the culture for 24hours or 3 hours before gelatinase activities analyses. Monensin (Sigma) at the concentration of 0.5 μM, 1μM and 5μM; pure TIMP-2 (Calibiochem) at the concentrations of 0.5ng/ml or 0.001ng/m were added to the culture medium for 24 hours before gelatinase analyses.

Zymography (25)

The proenzyme and activated forms of MMP-2 in the culture condition medium was detected by zymography using SDS-7% polyacrylamide gels copolymerized with 1 mg/mL gelatin. Sections of 5 μm/1 cm^2 were scraped off the slides. Protein contain was measured and equal amounts of samples were homogenized in sample buffer and directly electrophoresed. The gel was washed twice at room temperature for 10 minutes in 2.5% Triton X-100 and for 20 minutes in water and incubated overnight at 37℃ in 50 mmol/L Tris-HCL, pH 8, containing 5 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$. Gels was stained in 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant Blue G250. Both proenzyme and active proteinase forms were detected as clear bands and analyzed by densitometric scanning using a computer-assisted analysis (Alphalmager 2200).

Enzyme-linked immunoassays (ELISA)

A "sandwich: ELISA was established for the quantitative determination of the 72-kd MMP-2 proteins presented in the condition medium of culture cells. Matrix metalloproteinase-2 activity assay system (Amersham Pharmacia Biotech) will be used. 100μl of condition medium was added to each 96 well microtitre plate coated with anti MMP-2 and incubated overnight at 4℃.1 mM of p-Aminophenylmercuric acetate (APMA) was added into the microtitre plate when the active form of MMP-2 was to be detected, instead, assay buffer was added to detect the total form of MMP-2. Detection buffer containing modified urokinase and lyophilized S-2444™ peptide was added to the plate and incubated at 37℃for 1.5 hours. The resultant color was read at 405 nm in a microtitre plate spectrophotometer. All samples were measured in quadruplicate.

Results

Immunolocalization of MMP-2 and MMP-9 in ovarian tumor

Staining of MMP2 and MMP9 showed predominant at the cytoplasmic region and peripheral areas of tumor cells in clear cell carcinoma (20/21). In other pathological types of ovarian cancer (serous papillary adenocarcinoma, mucinous cystadenocarcinoma and endometriod carcinoma), MMP-2 and MMP-9 were either equally distributed in tumor cells and the subepithelial stromal cells (11/28 in MMP-2, 17/28 in MMP-9), or predominant in the stromal cells. In metastatic ovarian tumors (krukenberg tumor), MMP2 was equally distributed between the tumor and stromal cells (5/8); and MMP-9 was more predominant in the tumor cells (5/8). (Fig 1 and 2)

MMP-2 and TIMP-2 in situ hybridization

At present, 1 clear cell carcinoma and 1 serous papillary carcinoma adenocarcinoma showed MMP-2 and TIMP-2 mRNA distribution mainly localized at the tumor sites. (Fig 3 and 4). We are now continuing with this experiment.

Zymographic analysis of metalloproteiase activity

Condition mediums from ovarian carcinoma cell line OVTW59 were analyzed for MMP activity by zymography. Latent MMP-2 (72kD) and its active forms (64 and 62 kD), and MMP-9 (92 kD) were detected (Fig 5). Recombinant TGF-ß1 at the concentrations 0.5 to 5 ng /ml did not affect MMPs activities in OVTW-59. TIMP-2/MMP2 complex increased MMP2 (both latent and active forms) activities more predominant than MMP-9. These activities were higher after 3 hours reaction than 24 hours (Fig 6). Similar results were detected in 2 ovarian carcinoma primary cultures (both serous papillary adenocarcinoma) (data not shown).

Quantification of MMP-2 activity by ELISA

By using ELISA, we found similar effects of TGFß1 and TIMP2/MMP-2 complex on the MMP-2 activities in OVTW-59. Also, monensin at the concentrations of 0.5, 1 and 5 μM did not affect MMP-2 activites in OVTW-59. Pure TIMP-2 at the concentration of 0.5 ng increased MMP-2 activities slightly in OVTW-59 cultured of 24 hours (Fig 7).

Activities of MMPs by co-culture of primary ovarian culture cells and mesothelial cells

Three ovarian primary culture cells obtained form a clear cell carcinoma (O45), an

endometriod adenocarcinoma (O52), and a papillary serous adenocarcinoma (O57) were co-culture with a primary mesothelial cell culture obtained from a patient with borderline ovarian carcinoma. No difference in the activities of MMP-2 (Fig 8 A-C) and MMP-9 (Fig 8 D-F) were detected in zymography after co-cultures.

Activities of MMPs by co-culture of primary ovarian culture cells and human fibroblasts
Fibroblasts primary culture cells obtained from human scar tissues were co-culture with a primary ovarian carcinoma cell (O80) obtained from clear cell carcinoma at the ratio of 1:1 and 1:2. Fibroblasts cells were found to have profuse MMP-2 activity and no MMP-9 activities. MMP-2 activities were not affected by co-culture, but MMP-9 activities were activated during co-culture (Fig 9).

Discussion

The cellular source of MMP-2, whether if is of stromal or tumor cell origin remains ambiguous (26). Localization of MMP-2 by immunohistochemical studies was reported both in the stromal and epithelial tumor cells (11). In our studies, we found similar pattern of MMP-2 and MMP-9 distribution in ovarian carcinoma, except clear cell carcinoma. Other studies seldom included clear cell carcinoma (11,26). Absent of MMPs in the stromal cells therefore prove against to the theory that MMP was secreted from the stromal cells and bind to the ovarian cancer cell surface for activations (14). Ovarian clear cell carcinoma can secrete MMPs, or in the other way, it may lose its capability to stimulate its surrounding fibroblasts to secret MMPs. The cytoplasmic localization of MMPs in the ovarian carcinoma has been reported in multiple cancers (15). This may indicate internalization of the activated MMP-2 species, possibly by degradation of activated fragments or redistribution to different sites on the cell membrane.

Localization of mRNA of MMP-2 and TIMP2 was reported mainly in the stromal tissue of the ovaries (12,13). The only few cases of our in situ hybridization of MMP-2 and TIMP-2 however, showed localization of their mRNA in the tumor cells, similar as the protein distribution. We have difficulties in the development of the riboprobes, and were only recently established and succeed in the ISH experiments. We therefore spent more time on the following rest of the experiment and will continue the ISH in the following days.

High levels of MMP-2 was detected in ovarian cancer cells derived from ascitic fluid, primary ovarian tumors and metastatic lesions (10,16). We too found MMP-2 in the ovarian, mesothelial and fibroblast primary culture cells. All these cells, except fibroblasts, also secrets MMP-9. It was reported that TGF-ß are synthesed by human peritoneal mesothelial cells (27), and that TGF-ß1 can stimulate MMP-2. We were very interest to study the factor of TGF-ß1 on the metastatic mechanism of ovarian carcinoma. However, we did not find the effect of TGF-ß1 on the secretion of MMPs in ovarian carcinoma primary culture cells, and cell line. Monensin was used as negative control, and TIMP-2 as positive control. Interestingly, we found low dose of TIMP-2 stimulating the secretion of MMP-2. The complex form of TIMP-2 and MMP-2 further stimulating MMP-2 at short duration of interaction (3 hours). TIMP-2 is a natural inhibitor of MMP-2, but MMP-2 activation has been proposed through the trimolecular complex containing MT-MMP-1, TIMP-2 and MMP-2 (8). This thus explained our results.

Our results of MMPs activities in co-culture of primary ovarian carcinoma cells and mesothelial cells or fibroblasts were not as promising as reported (29,30). However, we did found elevation of MMP-9 with co-culture of ovarian clear cell carcinoma primary culture with fibroblasts. Repetition for more primary cultures is required for conformation. The possible explanation of the lack of reaction may be due to too short of co-culture duration

(24 hours), or to the inhibition effect of TIMP-2. Further modifications of the culture conditions are still undergoing.

Conclusion

We found an unique pattern of MMPs protein distribution in ovarian clear cell carcinoma from the other pathological types of ovarian epithelial cell carcinoma. Secretion of mRNA on MMPs are still undergoing and co-culture system in associate with zymography and ELISA technique may further clarify the mechanism of MMP activation in ovarian cancer.

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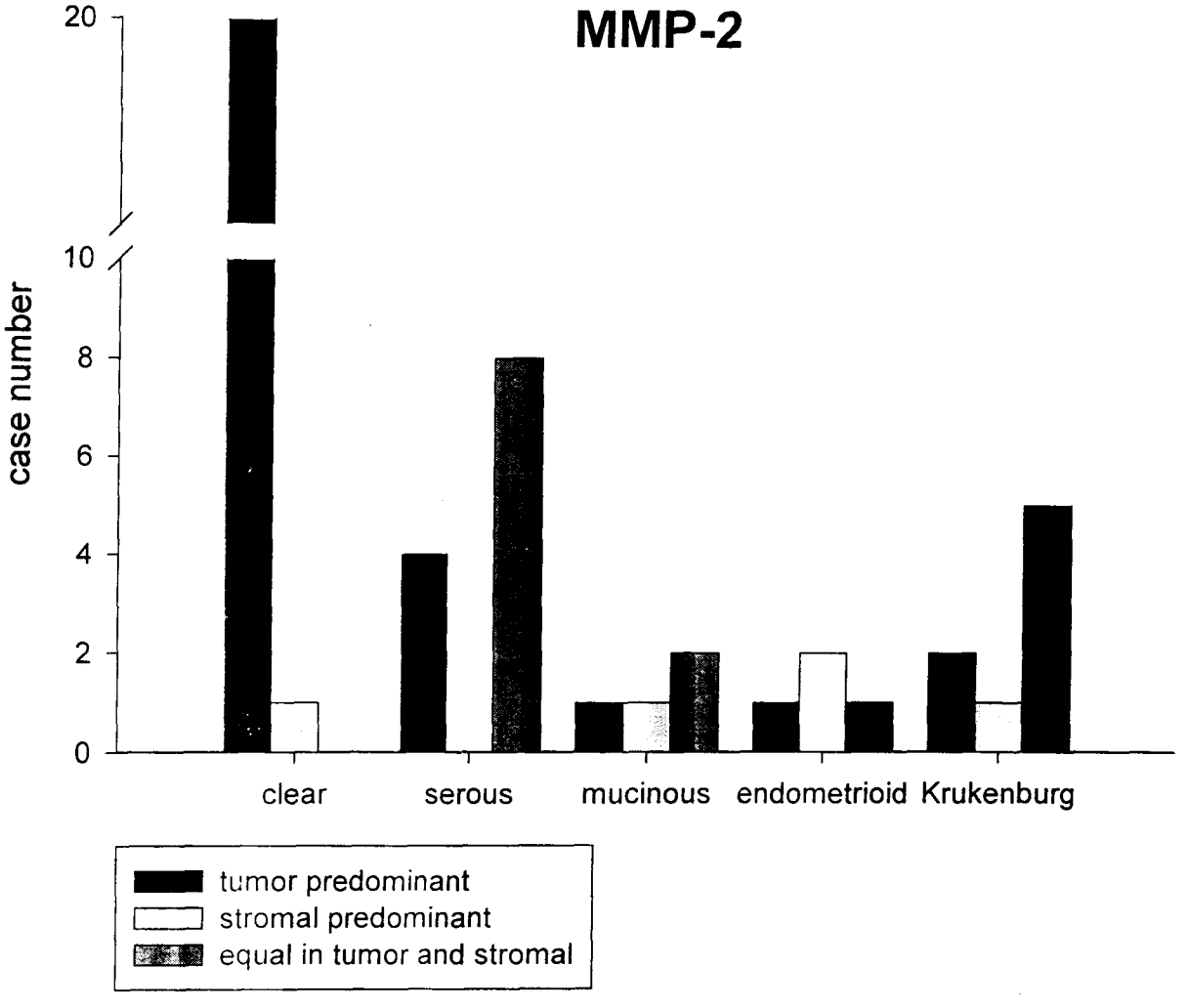

Fig 1. Immunolocalization of MMP-2 in ovarian tumors

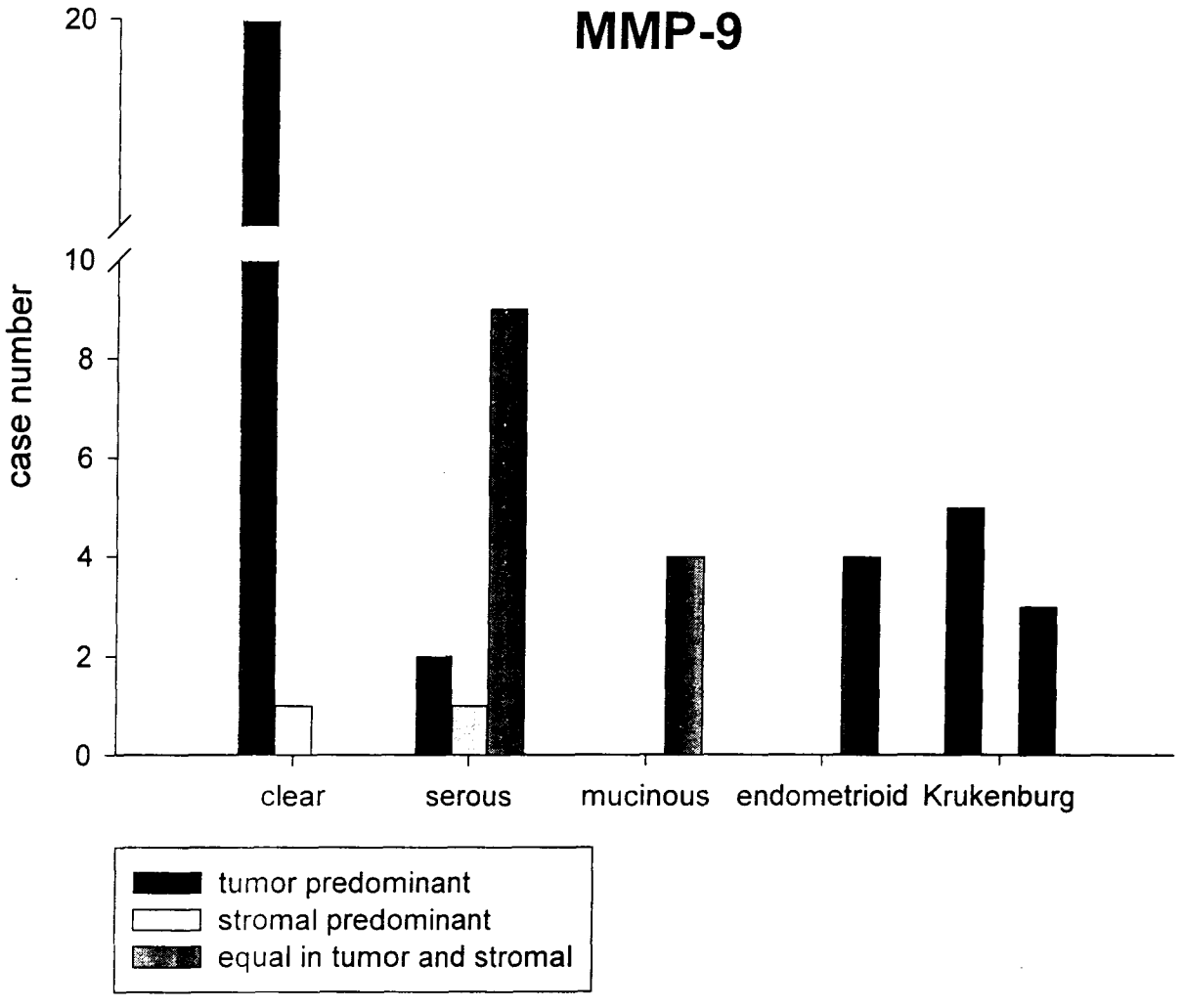

Fig 2. Immunolocalization of MMP-9 in ovarian tumors

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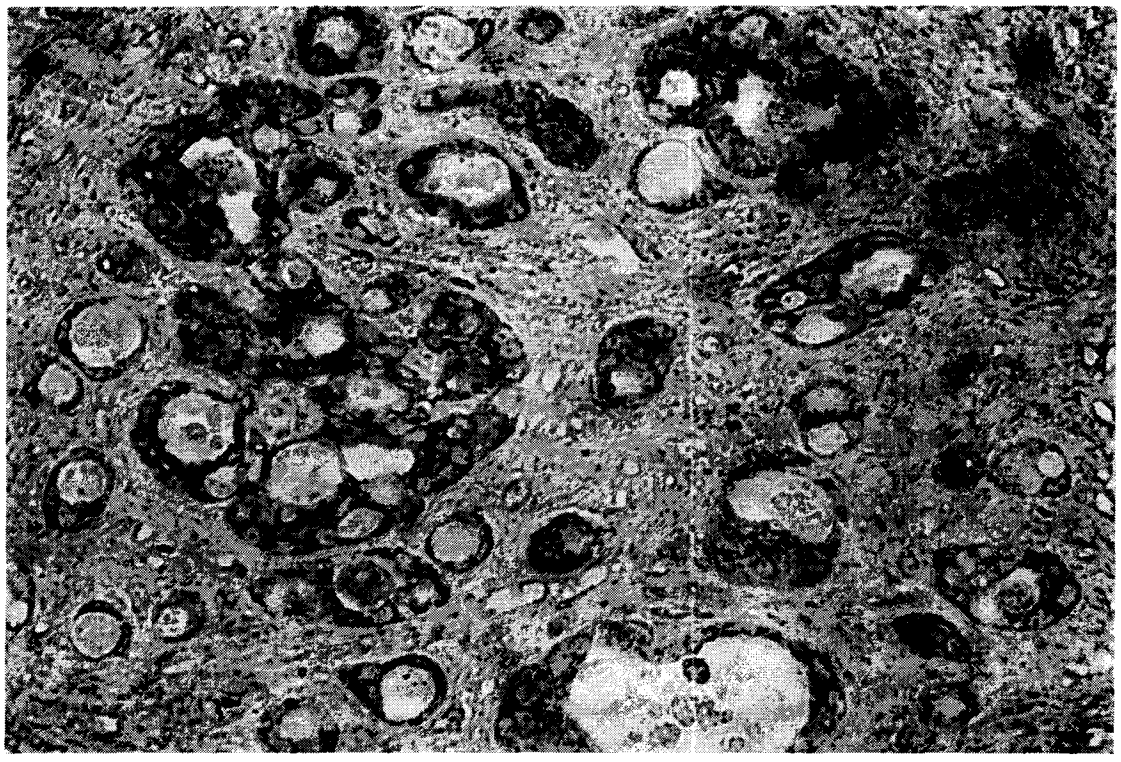

Fig 3 In situ hybridization of MMP-2 in clear cell carcinoma

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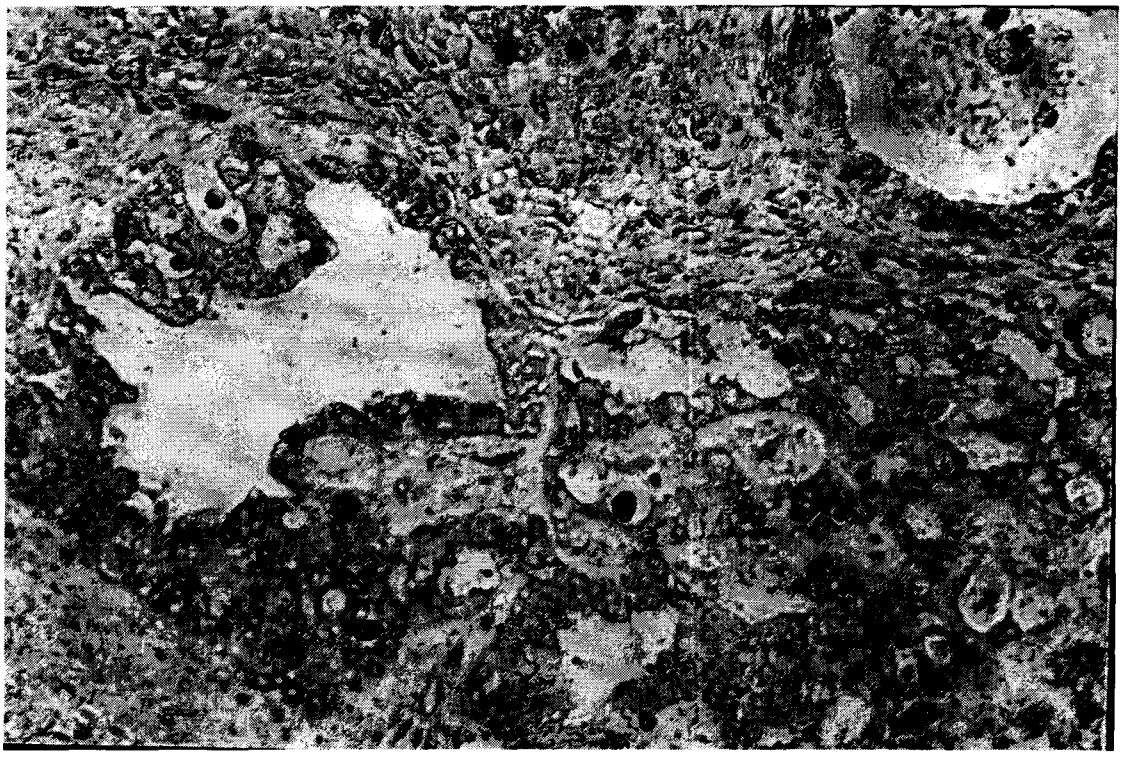

Fig 4. In situ hybridization of clear cell carcinoma

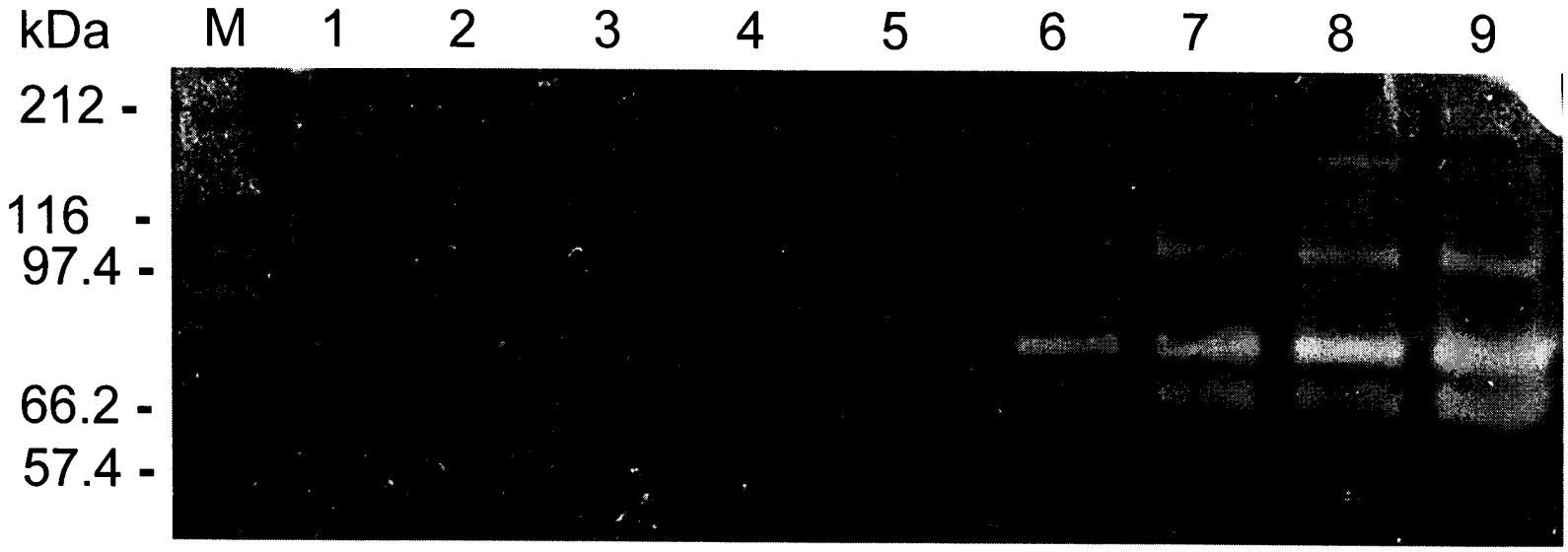

Fig 5 Zymographic analysis of MMPs activity in OVTW-59 cell line. Lanes: M: marker; 1: OVTW-59 culture alone;2: plus 0.5ng/mL TGF β 1 for 24 hr; 3: plus 1ng/mL TGF β 1 for 24 hr; 4: plus 2ng/mL TGF β 1 for 24 hr; 5: plus 5ng/mL TGF β 1 for 24 hr; 6: plus 0.5ng/mL TIMP-2 for 24 hr; 7: plus 1ng/mL TIMP-2 for 24; 8: plus 0.5ng/mL TIMP-2 for 3 hr; 9: plus 1ng/mL TIMP-2 for 3 hr.

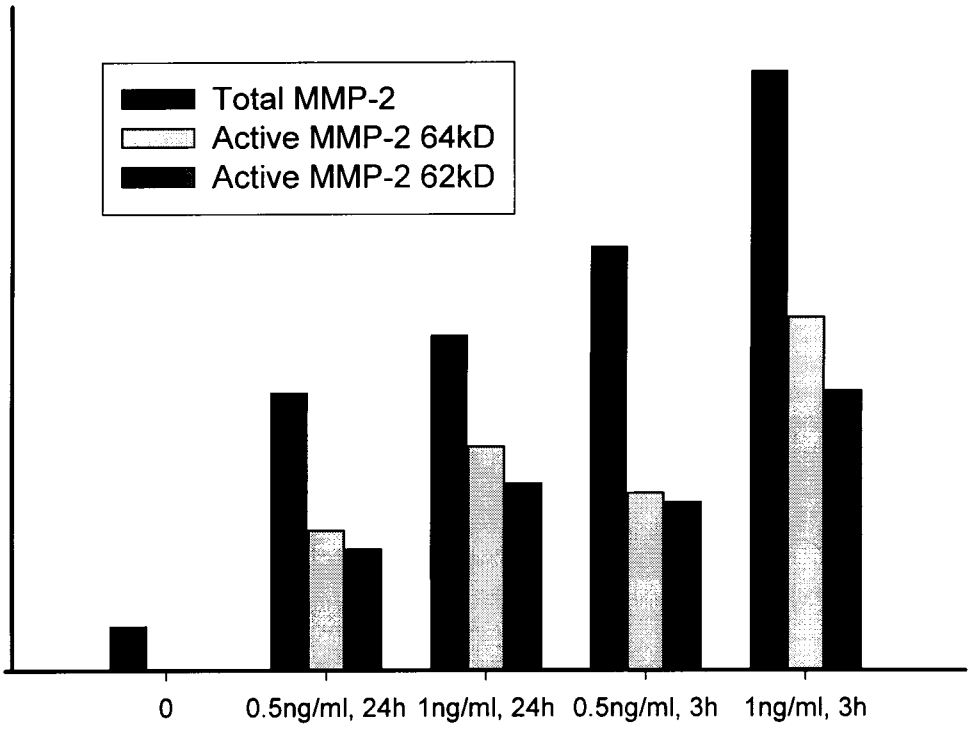

Fig 6. Densitomery of MMP2 activity by zymography. Activities of MMP-2 in OVTW-59 in different concentrations and culture times of TIMP-2/MMP-2 complex.

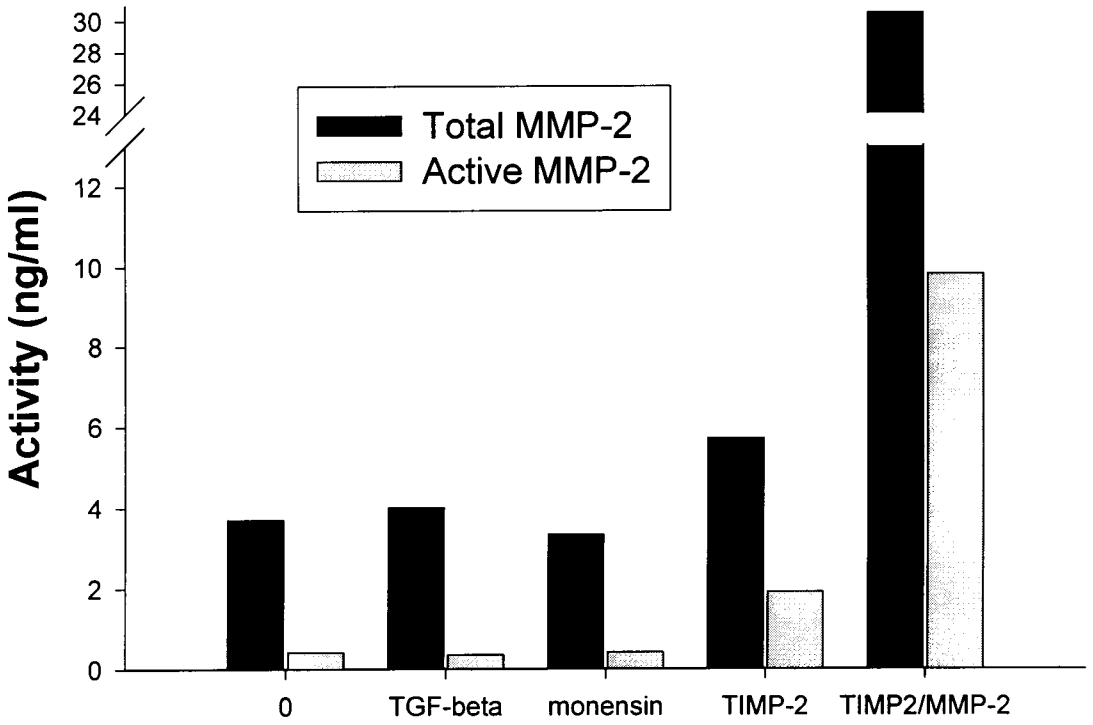

Fig 7. Quantification of MMP2 activity by ELISA. Activities of MMP-2 in OVTW-59 in control, 0.5 to 5 ng/ml TGF-beta 1, 0.5 to 5uM monensin, 0.5 ng/ml pure TIMP-2 and 0.5 ng/ml TIMP2/MMP-2 complex for 24 hours.

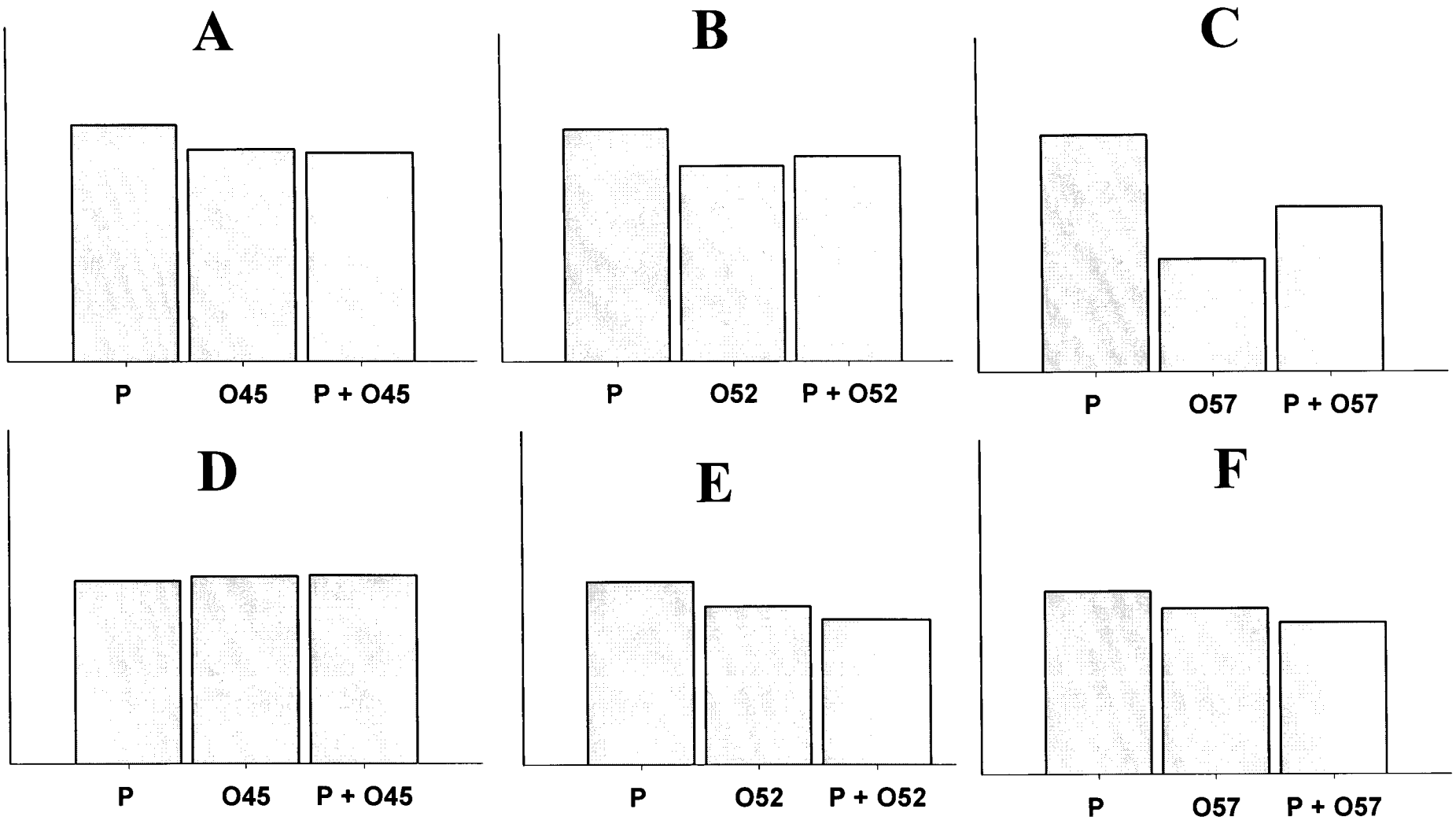

Fig 8 Avtivities of MMPs by co-culture of primary ovarian culture cells and mesothelial cells. A-C: activities of MMP-2; D-F: activities of MMP-9; P: mesothelium from the peritoneal tissue of a borderline ovarian carcinoma; O45: clear cell carcinoma; O52: endometriod carcinoma; O57: papillary serous adenocarcinoma.

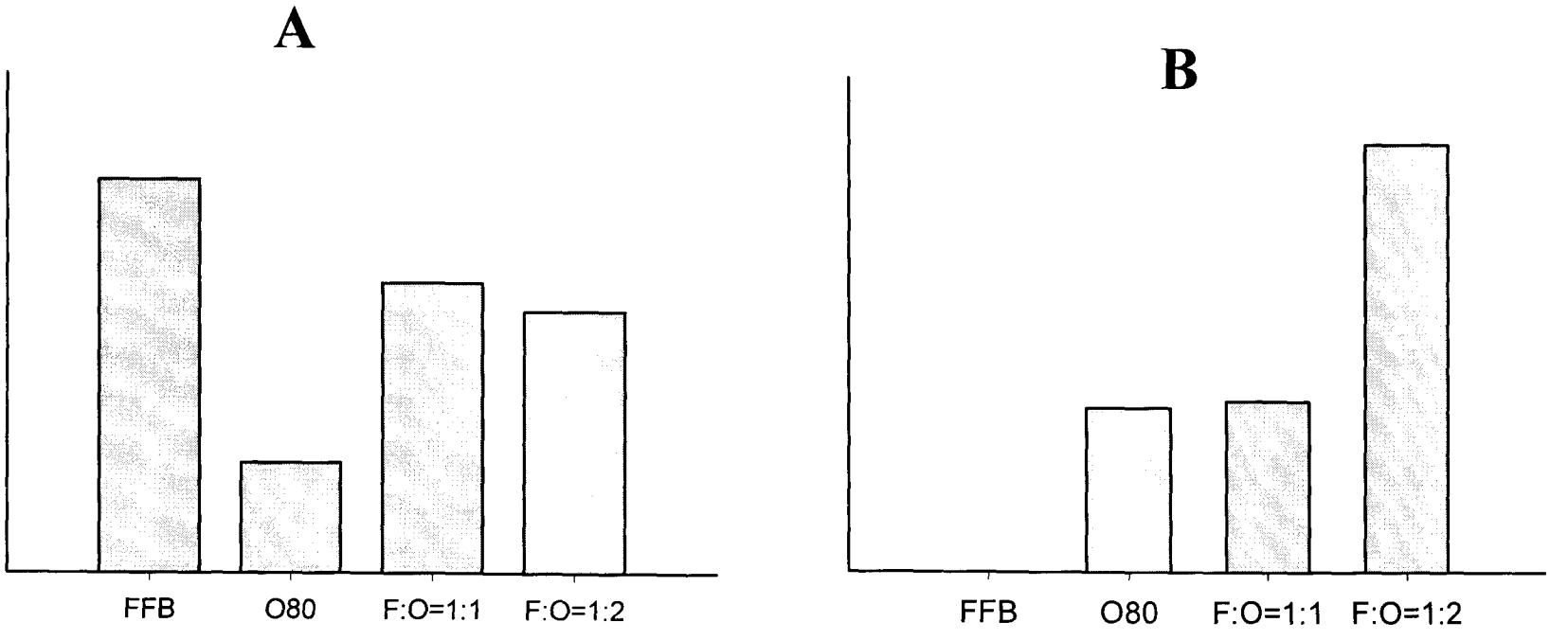

Fig 9 Activities of MMPs by co-culture of ovarian culture cells And human fibroblasts. A: activities of MMP-2; B: activities of MMP-9; FFB: fibroblasts; O80: primary culture of ovarian clear Cell carcinoma. (Zymography)