

# Increased Expression of Matrix Metalloproteinases (MMP)-2, MMP-9, and the Urokinase-Type Plasminogen Activator Is Associated with Progression from Benign to Advanced Ovarian Cancer<sup>1</sup>

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## ABSTRACT

Proteases are linked to the malignant phenotype of different solid tumors. Therefore, the expression of the matrix metalloproteinase (MMP)-2 and MMP-9 and of the serine protease urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) in the progression of ovarian cancer was investigated. Gelatinolytic activity and protein expression of MMP-2 and MMP-9 were analyzed in tissue extracts of 19 cystadenomas and 18 low malignant potential (LMP) tumors, as well as 41 primary tumors of advanced ovarian cancer stage International Federation of Gynecology and Obstetrics IIIc/IV and their corresponding omentum metastases by quantitative gelatin zymography and Western blot. In the same tissue extracts, antigen levels of uPA and its inhibitor PAI-1 were determined by ELISA. Protein expression of pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) as well as antigen levels of uPA and PAI-1 were low in benign ovarian tumors but increased significantly from LMP tumors to advanced ovarian cancers. The highest values of all of the proteolytic factors were detected in omentum metastases. Active MMP-2 enzyme (62 kDa) was detected only in

ovarian cancer (66%) and corresponding metastases (93%) but never in benign or LMP tumors. The activation rate of MMP-2 to its active isoform was higher in the metastases. Comparing both proteolytic systems, higher PAI-1 concentrations were consistently found in cancers with high pro-MMP-9 expression. These data indicate that members of the plasminogen activator system, as well as the metalloproteinases MMP-2/9, increase with growing malignant potential of ovarian tumors. These findings are of particular relevance to the development of protease inhibitors as new therapeutic approaches in ovarian cancer.

## INTRODUCTION

The metastatic process involves intravasation and extravasation of tumor cells, followed by reimplantation of tumor cells, formation of a new tumor stroma, and neoangiogenesis to consolidate a secondary tumor at a distant site (1). Degradation of the extracellular matrix and components of the basement membrane by proteases facilitates the detachment of tumor cells, their crossing of tissue boundaries, and invasion into adjacent tissue compartments. In recent years, the importance of tumor-associated proteases in invasion and metastasis has been demonstrated for a variety of solid malignant tumors. The serine protease uPA<sup>3</sup>, its inhibitor PAI-1, and the MMPs are instrumental in these processes (2). uPA facilitates extracellular matrix degradation by converting the zymogen plasminogen into plasmin, a serine protease with broad substrate specificity. It binds to a highly glycosylated receptor (uPAR, CD87) that focuses the proteolytic activity to the cell surface. The uPA inhibitor PAI-1 regulates uPA activity and plays an important role in reimplantation of disseminated tumor cells and formation of a new tumor stroma at the site of the metastasis. Besides its role in proteolysis, the plasminogen activation system stimulates cell proliferation and modulates cell adhesion (3, 4).

Similar to the plasminogen activator system, members of the MMP family have been implicated in tumor cell invasion and metastasis. MMPs are characterized by a zinc atom at the active site and are classified according to homologies in sequence and substrate affinity (5). The two metalloproteinases, MMP-2 and MMP-9 (72 kDa and 92 kDa type IV collagenases or gelatinase A and gelatinase B, respectively), have been associated with the malignant phenotype of tumor cells because of their unique ability to degrade type IV collagen, which is a

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<sup>3</sup> The abbreviations used are: uPA, urokinase-type plasminogen activator; LMP, low malignant potential; MMP, matrix metalloproteinase; uPAR, urokinase-type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor type I; FIGO, International Federation of Gynecology and Obstetrics; APMA, aminophenyl-mercuric acetate.

major component of the basement membrane (6). One of the first observations that suggested a role for MMP-9 in tumor invasion relates to the fact that the release of MMP-9 is associated with the metastatic phenotype of transformed rat embryo cells (7). For ovarian cancer, secretion of MMP-2 and MMP-9 has been observed in several ovarian cancer cell lines and detected in ascitic fluid from patients with advanced ovarian cancer. The invasiveness of ovarian cancer cell lines correlated with expression of MMP-2 and MMP-9 *in vitro* (8–11). Naylor *et al.* (12) reported elevated MMP-9 levels in ovarian cancers as compared with breast and bladder cancer. Recently, in an immunohistochemical study (13) encompassing 33 malignant ovarian lesions, positive staining for MMP-2 was associated with poor survival, but in two other studies (14, 15) MMP-2 expression had no prognostic impact on survival. These authors did not analyze whether the enzymatic activity of MMP-2 differs in benign ovarian tumors from malignant ovarian tumors. Even more, no data on MMP-9 expression or activity in the various steps of ovarian cancer progression have been reported.

Because MMP-2/9 and uPA are key factors in the promotion of tumor-associated proteolysis and we and others have shown previously (16–19) that uPA is important for ovarian cancer progression, we undertook a study with two objectives: (a) to analyze MMP-2 and MMP-9 protein expression and activity in tissue extracts of benign tumors (cystadenomas), atypically proliferating tumors of LMP tumors, and primary tumors of advanced ovarian cancers FIGO IIIc and IV and their corresponding omentum metastases and (b) to compare MMP-2/9 with uPA and PAI-1 antigen levels in the same tumor tissues.

## PATIENTS AND METHODS

**Patients and Tumor Samples.** Eighteen patients with ovarian LMP tumors and 41 patients with advanced ovarian cancer stage FIGO IIIc or IV were enrolled in a study on ovarian cancer performed at the Department of Obstetrics and Gynecology at the Technische Universität München. All of the patients gave informed consent, and the study was approved by the local ethics committee. All of the patients with LMP tumors were alive at the time of follow-up (2000). Twenty-eight (68%) of 41 patients with advanced ovarian cancer FIGO IIIc/IV died of the disease. Clinical and histomorphological data including median observation time of patients alive, median age, tumor stage, histology, nodal status, and ascites volume are given in Table 1. Therapy for all of the patients with invasive ovarian cancer consisted of radical surgical debulking followed by chemotherapy with platinum compounds and/or paclitaxel. In LMP tumors, less radical treatment was performed to preserve fertility. Control tissue extracts were prepared from 19 benign ovarian tumors (serous cystadenomas) of postmenopausal women (median age, 68 years; range, 44–87 years).

**Tissue Collection and Extraction.** Tissue samples were collected during surgery, and cryostat sections were examined by the pathologist and then stored in liquid nitrogen for further analysis. Ovarian tissue was pulverized in the frozen state by a micro-dismembrator (Braun-Melsungen, Melsungen, Germany) and resuspended in a buffer containing 0.02 M Tris-HCl (pH 8.5), 0.125 M NaCl, and 1% Triton X-100 under gentle rotation

**Table 1** Clinical data of patients with LMP tumors ( $n = 18$ ) and advanced ovarian cancer FIGO IIIc/IV ( $n = 41$ )

	LMP tumors ( $n = 18$ )	Ovarian cancer FIGO IIIc/IV ( $n = 41$ )
Median age (range)	52.5 (24–88) yr	60 (45–77) yr
Median observation time of patients alive (range)	41 (11–78) months	58 (12–100) months
	$n$ (%)	$n$ (%)
FIGO stage		
Ia	14 (78)	
Ib	1 (5)	
Ic	3 (17)	
IIIc		27 (66)
IV		14 (34)
Histology		
Serous	12 (67)	24 (58.5)
Mucinous	5 (28)	3 (7)
Endometrioid		4 (10)
Clear cell	1 (5)	2 (5)
Undifferentiated		8 (19.5)
Grading		
G <sub>1</sub> + G <sub>2</sub>		9 (22)
G <sub>3</sub> + G <sub>4</sub>		32 (78)
Nodal status		
N <sub>0</sub>	10 (56)	9 (22)
N <sub>1</sub>		20 (49)
N <sub>x</sub>	8 (44)	12 (29)
Ascites volume		
≤500 ml	18 (100)	14 (34)
>500 ml		27 (66)
Residual tumor		
Absent	18 (100)	12 (29)
Present		29 (71)
Chemotherapy		
Platinum and/or paclitaxel	2 (11)	35 (85)
Other	1 (6)	4 (10)
None	15 (83)	2 (5)

at 4°C. The lysate was ultracentrifuged at 100,000 × *g* (45 min; 4°C), and an aliquot of the supernatant was assayed for protein concentration using the BCA Protein Assay reagent kit (Pierce, Rockford, IL; Refs. 16, 17).

**uPA and PAI-1 ELISA.** uPA and PAI-1 antigen concentrations were determined by ELISA kits Imubind 894 and 821 (American Diagnostica, Greenwich, CT). Protein content of the tissue extracts was determined. uPA and PAI-1 antigen values were expressed as ng/mg protein (16, 17).

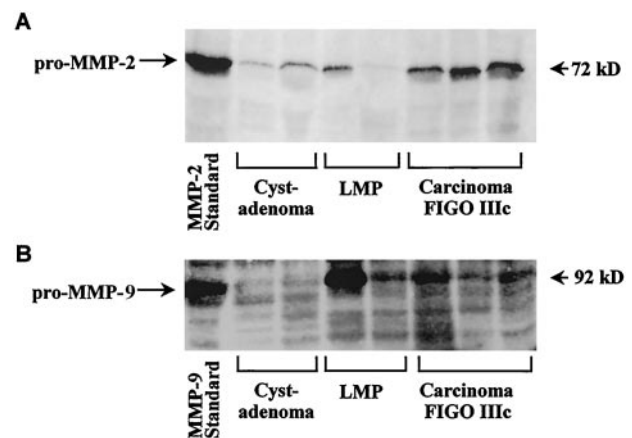
**Western Blot.** After extraction, tumor samples were adjusted to an equal amount of protein (100 μg), electrophoresed in a 12.5% SDS-PAGE, and transferred to a nitrocellulose membrane (BA-S85; Schleicher & Schuell, Dassel, Germany) by electroblotting. The membrane was blocked with 0.25% NET-gelatin [150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 0.05% Triton X-100] and sequentially incubated with a 1:3000 dilution of the monoclonal antibody #209 against human MMP-9 followed by a horseradish peroxidase-conjugated antimouse IgG. Reactive proteins were visualized by a chemiluminescence detection system (enhanced chemilumi-

nescence; Amersham, Buckinghamshire, United Kingdom). The blot was stripped for 1 h at 50°C with a solution containing 65 mM Tris (pH 6.8), 2% SDS, 100 mM  $\beta$ -mercaptoethanol, washed with NET gelatin, and then incubated with a 1:3000 dilution of the monoclonal antibody anti-MMP-2 RP1G72 against human MMP-2 (Triple Point Biologicals, Forest Grove, Oregon). As a positive control, in each gel 25 ng of recombinant human MMP-2 and MMP-9 (DIABOR, Oulo, Finland) was included. Protein expression levels of MMP-2 and MMP-9 in ovarian tissue samples relative to the standards were determined by linear densitometric scanning and expressed as ng/ $\mu$ g protein.

**Gelatin Zymography.** MMP-2 and MMP-9 enzymatic activity in tissue extracts was determined by SDS-PAGE gelatin zymography. Gelatinases present in the tissue extracts degrade the gelatin matrix, leaving a clear band after staining the gel for protein (21). Briefly, homogenized tissue samples, normalized to an equal amount of protein (40  $\mu$ g), were denatured in the absence of a reducing agent and electrophoresed in 7.5% SDS-PAGE containing 0.1% (w/v) gelatin. Gels were incubated in the presence of 2.5% Triton X-100 at room temperature for 2 h and subsequently at 37°C over night in a buffer containing 10 mM  $\text{CaCl}_2$ , 0.15 M NaCl, and 50 mM Tris (pH 7.5). Thereafter, gels were stained with 0.25% Coomassie Blue, and proteolysis was detected as a white band against a blue background. Every gel was included as a standard 3.125/6.25/12.5/25 ng of recombinant human MMP-2 and MMP-9 (DIABOR). Every zymogram was run twice. The activity of MMP-2 and MMP-9 relative to the standards was determined by densitometric scanning of the bands (Epson GT-9500 scanner) and analysis by Scan Pack 3.0 software (Biometra, Göttingen, Germany). In contrast to the Western blots that were scanned by linear densitometry, the zymograms were quantified by integrated density, taking intensity and width of the band into consideration (22). Gelatinolytic MMP-2 and MMP-9 activity is given in arbitrary units (units/ $\mu$ g protein). To investigate whether pro-MMP-2 and MMP-9 can be activated in human ovarian tissue, samples were preincubated with APMA (Sigma Chemical Co.), which activates the pro-form to the activated form.

**Immunohistochemistry.** Immunohistochemical staining for MMP-9 protein was performed by an avidin-biotin peroxidase technique on paraffin-embedded, formalin-fixed sections using the DAKO ChemMate Detection Kit 3,3'-diaminobenzidine (DAKO, Hamburg, Germany) as described (23). The mouse monoclonal MMP-9 antibody RP1G92 (10  $\mu$ g/ml; Triple Point Biologicals) or the monoclonal antibody #209 (20) against MMP-9 were each incubated for 2 h at RT. Specificity of the MMP-9 staining was assessed by the use of irrelevant control antibodies. No signals were obtained in these reactions.

**Statistical Analysis.** All of the results are given as median values. The significance level of differences between the antigen levels in cancer tissue extracts *versus* LMP tumors and cystadenomas was evaluated using the Mann-Whitney *U* test and the Kruskal-Wallis test, respectively, according to the rules for multiple hierarchical testing. Differences in antigen concentrations between specimens of the same patient were tested for significance using the Wilcoxon test for combined samples. Correlations between continuous variables were described using the Spearman correlation coefficient. Differences were considered to be statistically significant at a level of  $P < 0.05$ .

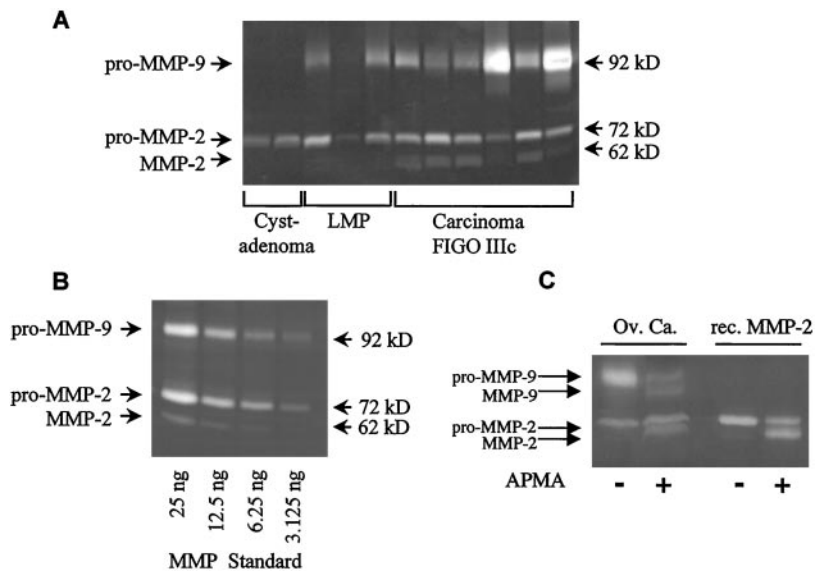


**Fig. 1** Western blot analysis of protein expression of (A) pro-MMP-2 (72 kDa) and (B) pro-MMP-9 (92 kDa) in tissues of ovarian serous cystadenomas, LMP tumors, and advanced ovarian cancers FIGO IIIc/IV. Recombinant MMP-2 and MMP-9 proteins (25 ng) served as standard.

## RESULTS

**MMP-2 and MMP-9 Protein Expression in Benign and Malignant Ovarian Tumors.** MMP-2 and MMP-9 protein expression was analyzed by Western blot in 19 ovarian cystadenomas and 18 LMP tumors, as well as in 41 ovarian cancers and their corresponding metastases (Fig. 1). The 12.5% SDS-PAGE resolved the latent proenzyme (72 kDa) and the enzymatically active form (62 kDa) of MMP-2 when assaying recombinant MMP-2 (data not shown), but active MMP-2 was never detected by Western Blot in ovarian tissue. This was caused by the low expression of the active form of MMP-2 in ovarian tissue, which could only be detected with the more sensitive zymogram (Fig. 2). Median pro-MMP-2 (72 kDa) protein expression as determined by Western Blot was low in cystadenomas (0.47 ng/ $\mu$ g) but increased significantly from LMP tumors (0.78 ng/ $\mu$ g protein) to advanced ovarian cancer FIGO IIIc and IV (1.20 ng/ $\mu$ g protein; Fig. 3A). There was a trend toward higher pro-MMP-2 (72 kDa) expression in the omentum metastases (1.43 ng/ $\mu$ g protein) than in the primary tumors, but the difference was not statistically significant. Median pro-MMP-9 (92 kDa) protein expression increased gradually from benign ovarian tumors (0.29 ng/ $\mu$ g protein) over LMP tumors (0.38 ng/ $\mu$ g protein) to advanced ovarian cancer FIGO IIIc/IV (0.57 ng/ $\mu$ g protein; Fig. 3B). Median pro-MMP-9 concentration in primary ovarian cancer extracts was twice as high than that in ovarian cystadenomas. An increase in median pro-MMP-9 expression in omentum metastases (0.68 ng/ $\mu$ g protein) compared with the expression in primary tumors was noted, but this was not statistically significant. Active MMP-9 (82 kDa) could not be detected by Western blotting either in benign or in malignant ovarian tissues.

**Enzymatic Activity of MMP-2 and MMP-9 in Benign and Malignant Ovarian Tumors.** The expression of MMP-2 and MMP-9 was also measured by gelatin zymography of the same tumor specimens (Fig. 2, A and B). This technique resolves the gelatinases by their molecular mass and is appropriate for detection of both the active and the latent forms of the enzymes



**Fig. 2** Zymograms. **A**, analysis of gelatinolytic activity in tissues of ovarian serous cystadenomas, LMP tumors, and advanced ovarian cancers; and **B**, different concentrations of recombinant MMP-2 and MMP-9 proteins for a standard curve. **C**, tissue extract from an ovarian cancer and recombinant MMP-2 was incubated with or without APMA for 1 h at 37°C and then subjected to zymography.

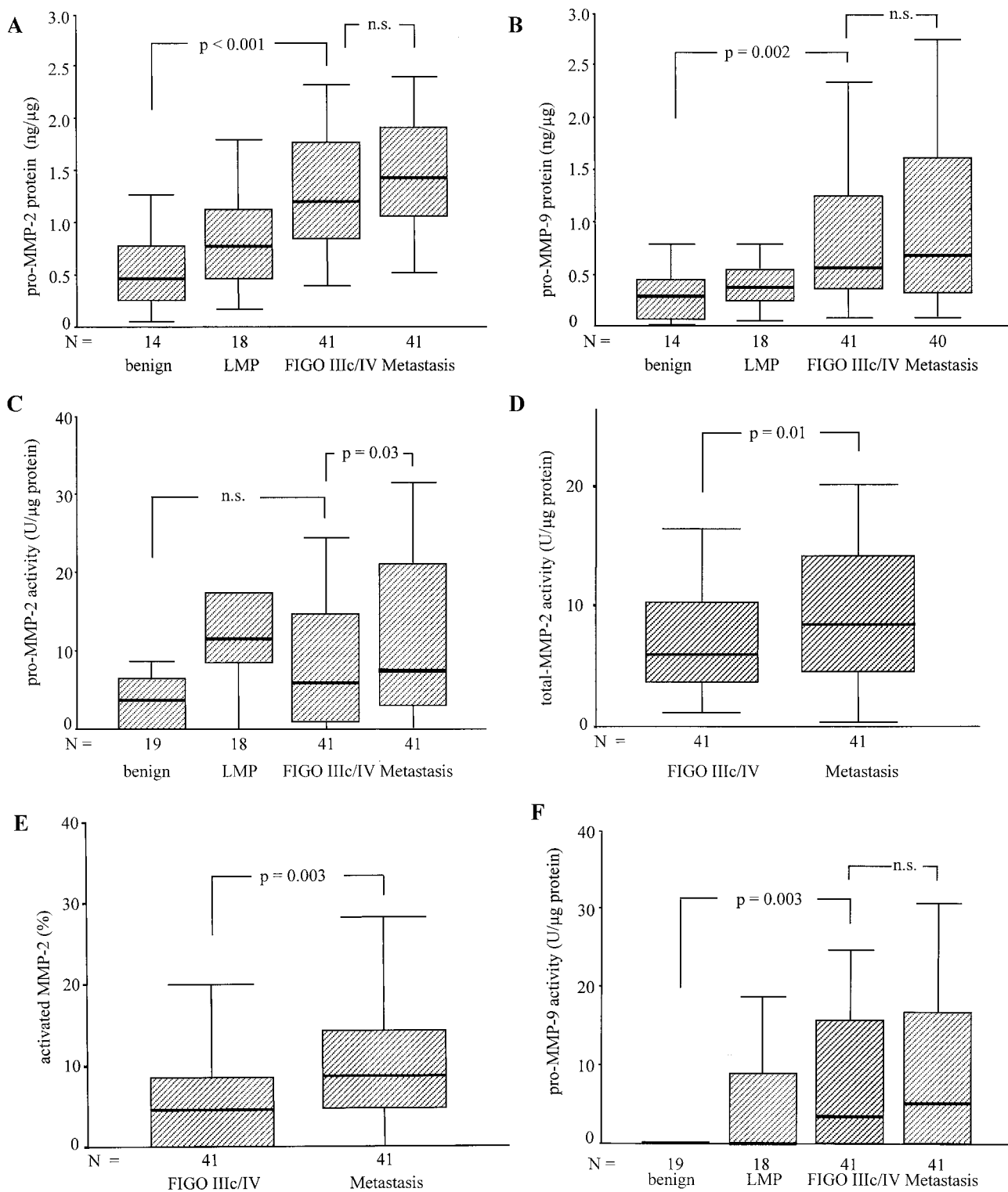
because in the presence of the detergent SDS, the enzymes are denatured, exposing their active site, which permits both the latent and active forms of the gelatinases to exhibit gelatinolytic activity after partial renaturation (24). Median pro-MMP-2 (72 kDa) activity was slightly higher in primary tumor tissue from advanced ovarian cancer (5.96 units/ $\mu$ g) than in benign ovarian tumors (3.70 units/ $\mu$ g); however, this difference was not statistically significant (Fig. 3C). In tumor-infiltrated omentum majus of advanced ovarian cancer, a statistically significant increase in pro-MMP-2 (7.52 units/ $\mu$ g protein) compared with the corresponding primary tumor was noted. Interestingly, the highest pro-MMP-2 expression (11.5 units/ $\mu$ g protein) was found in LMP tumors. There was a significant difference in the presence of active MMP-2 (62 kDa) between benign ovarian tumors and advanced ovarian cancer (Fig. 2A). No active MMP-2 could be detected by zymography in any of the 19 benign ovarian tumors or in the 18 cases of LMP tumors; however, active MMP-2 (62 kDa) was present in 27 (66%) of the primary tumors and in 38 (93%) of the omentum metastases of 41 advanced ovarian cancers. The median of total MMP-2 activity (pro-MMP-2 + active MMP-2) between primary tumor (6.19 units/ $\mu$ g) and omental metastases (8.5 units/ $\mu$ g) was significantly different (Fig. 3D). When the intensity of the proteolytic bands of pro-MMP-2 and its active species was compared, in primary tumors 4.5% (median) of total MMP-2 was active MMP-2, whereas in the metastases, 8.7% of total MMP-2 was activated ( $P = 0.003$ ; Fig. 3E). This suggests that the tumor cells that are able to metastasize have a higher activation ratio of pro-MMP-2:active MMP-2.

With respect to MMP-9, a significant rise in median pro-MMP-9 (92 kDa) activity from benign ovarian tumors to advanced ovarian cancer was observed in the zymograms. Whereas no pro-MMP-9 activity was detected in ovarian cystadenomas and median expression in LMP tumors was zero, primary ovarian cancers expressed pro-MMP-9 with a median of 3.48 units/ $\mu$ g protein, which did not further increase in the

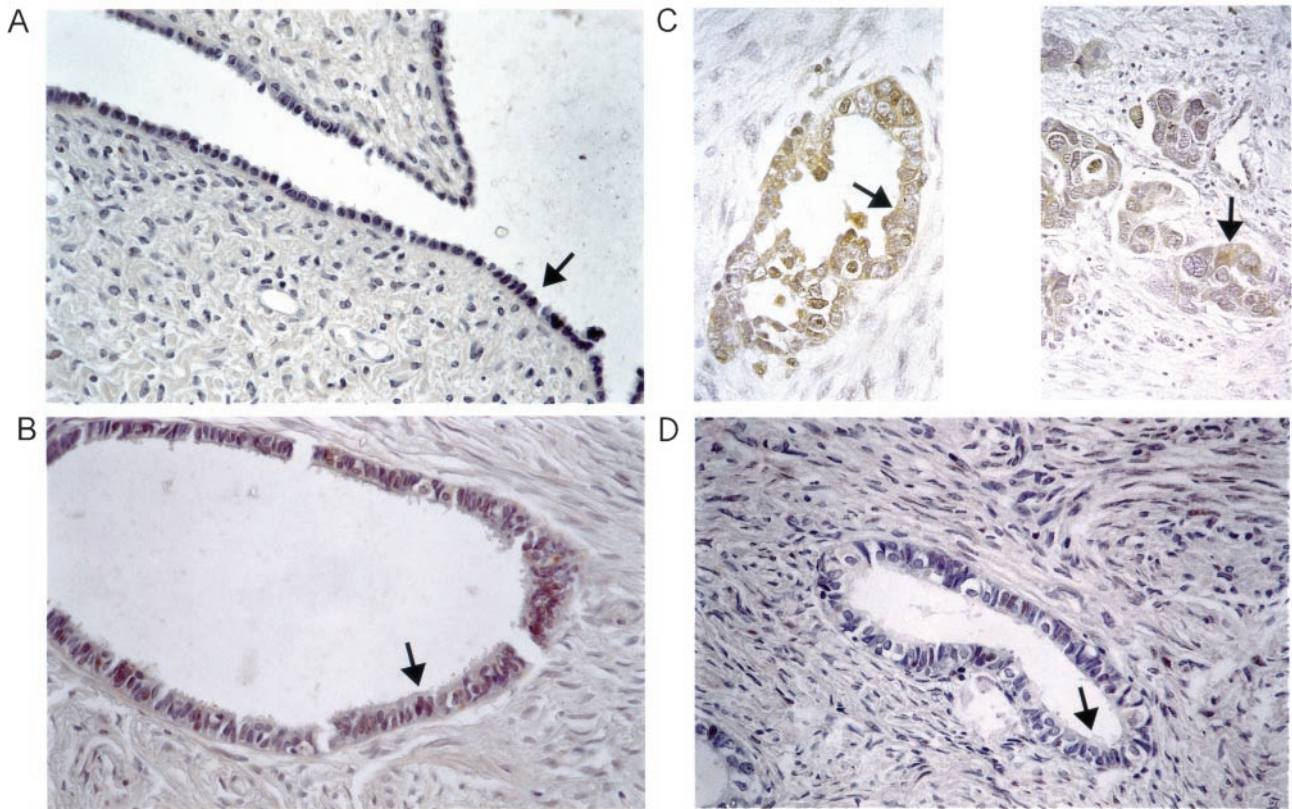
corresponding metastases (Fig. 3F). Like in Western blot analyses, none of the ovarian tissues displayed the active 82 kDa form of MMP-9 by zymographic analyses (Fig. 2A). Preincubation of an ovarian cancer tissue that showed only pro-MMP-9 and pro-MMP-2 activity with APMA resulted in conversion of pro-MMP-9 in the tumor to a smaller form of 82 kDa size, as expected for the active form of MMP-9 and conversion of pro-MMP-2 to a smaller form of about 68 kDa. The smaller band comigrated with APMA-activated recombinant MMP-2, suggesting that it is active MMP-2 (Fig. 2C).

**Immunohistochemical Staining of MMP-9 in Benign and Malignant Ovarian Tumors.** Because the results with Western blot and zymography indicated an increase in pro-MMP-9 protein expression and activity from benign to malignant ovarian tumors, immunohistochemical staining was performed to determine the expression pattern of MMP-9. As shown in Fig. 4, MMP-9 staining could not be detected in ovarian cystadenoma (Fig. 4A), whereas moderate staining in the cytoplasm of tumor cells was observed in LMP tumors (Fig. 4B). Strong staining was detected in primary ovarian cancer and its corresponding metastases (Fig. 4C). The main staining intensity was located in the epithelial tumor cell compartment. In addition, a weak staining was also observed in some stromal cells. Incubation with normal mouse IgGs yielded no staining (Fig. 4D).

**Antigen Concentrations of uPA and PAI-1 in Benign and Malignant Ovarian Tumors.** The antigen content of uPA and its inhibitor PAI-1 in ovarian tumor tissue extracts was determined by ELISA and correlated to MMP-2 and MMP-9 protein expression measured in the same tissue extracts (Fig. 5, A and B). Median uPA and PAI-1 antigen concentrations increased from cystadenomas (0.24 ng/mg and 4.50 ng/mg, respectively) over LMP tumors (0.54 and 6.93 ng/mg protein, respectively) to primary tumors of advanced ovarian cancer (1.14 and 15.40 ng/mg protein, respectively). Median concentration of uPA in the omentum metastases (3.91 ng/mg protein)



**Fig. 3** Box blots of protein expression as determined by Western blot of (A) pro-MMP-2 (72 kDa) and (B) pro-MMP-9 (92 kDa). C, gelatinolytic activity as determined by zymographic analysis of pro-MMP-2 in tissue extracts of ovarian serous cystadenomas, LMP tumors, FIGO IIIc/IV cancers, and corresponding metastases. D, total MMP-2 (pro-MMP-2 + activated MMP-2) and E, percentage of activated MMP-2 protein (68 kDa) in FIGO IIIc/IV ovarian cancer and corresponding metastases, as measured by zymography. The *bottom* boundary of the box plot represents the value of the 25<sup>th</sup> percentile; the *top* boundary represents the value of the 75<sup>th</sup> percentile. The *bars* indicate minimum and maximum values without extremes. Median values are marked within the boxes by *bold horizontal bars*. Statistically significant differences between groups are indicated by *Ps*.



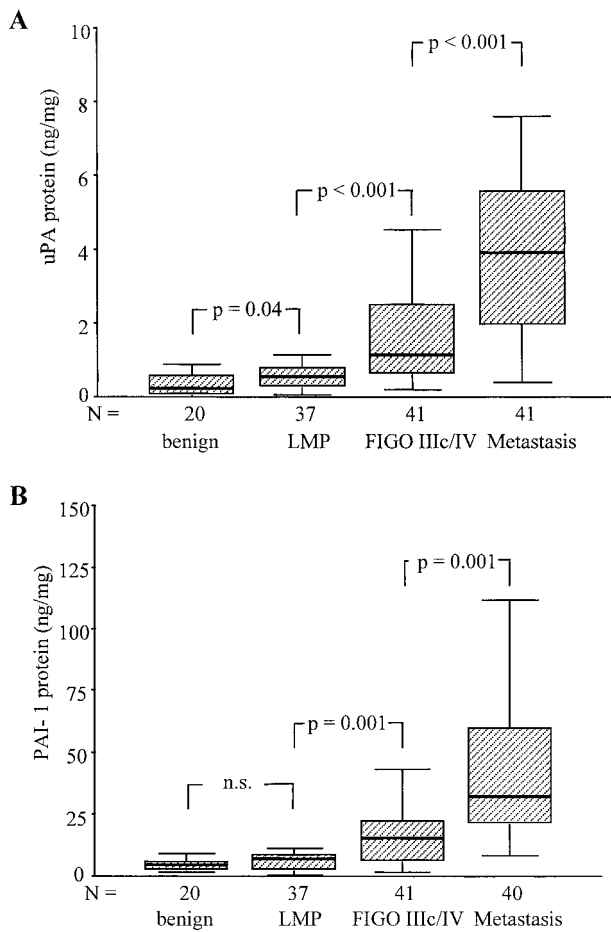
**Fig. 4** Immunohistochemical staining (brown) for MMP-9 (anti-MMP-RP1G92). Nuclei are stained with hematoxylin. **A**, serous ovarian cystadenoma. Note there is no staining in the cytoplasm of the tumor cells (arrow). Only some stromal cells show light staining. **B**, serous ovarian LMP tumor. The cytoplasm of the tumor cells stains positive for MMP-9 (arrow). **C**, serous ovarian cancer FIGO IV. *Left panel*, primary tumor; *right panel*, omentum metastases. Positive staining of the cytoplasm in the tumor cells (arrow). **D**, control staining of the LMP tumor with an equivalent concentration of normal mouse IgGs yields no staining in the epithelial cells (arrow). Original magnification (A–D),  $\times 200$ .

was three times higher and that of PAI-1 (32.34 ng/mg protein) was two times higher than that of the corresponding primary tumor.

**Correlations Between uPA, PAI-1, MMP-2, and MMP-9 in Primary Ovarian Cancer.** Spearman correlations for uPA, PAI-1, MMP-2, and MMP-9 were calculated in the group of advanced ovarian cancer FIGO IIIc/IV. Protein expression and enzymatic activity of pro-MMP-2 showed a statistically significant correlation ( $P = 0.004$ ;  $r = 0.44$ ), similar to pro-MMP-9 protein expression and enzymatic activity that were also correlated ( $P = 0.04$ ;  $r = 0.33$ ). The unsuspected low correlation between pro-MMP-2 determined by Western blot and pro-MMP-2 determined by zymography is probably attributable to the different sensitivities of the two methods and a difference in assessing the bands. Although zymograms have been quantified by taking into account the intensity and the width of the band as suggested (25–27), Western blots have been quantified by linear densitometric scanning, measuring only the intensity of the band (25). Regarding PAI-1, there was a correlation with pro-MMP-9 determined by zymography ( $P = 0.003$ ;  $r = 0.45$ ). Pro-MMP-2 measured by zymography was high in some PAI-1 high tumors; however, the correlation coefficient was low. No association was noted between uPA and MMPs.

## DISCUSSION

This study describes the distribution of MMP-2, MMP-9, and uPA, as well as its inhibitor PAI-1, in normal ovarian tissue, LMP tumors, advanced ovarian cancers, and their corresponding omental metastases. Using Western blot, ELISA, and zymograms, we show that pro-MMP-2, pro-MMP-9, uPA, and PAI-1 protein expression increases from benign to malignant ovarian tumors and that active MMP-2 is only detectable in ovarian cancer and increases further in metastases. Our findings confirm previous studies on pro-MMP-2 protein expression by Campo *et al.* (28) who by immunohistochemistry found no or only minimal expression of MMP-2 in cystadenomas but moderate to intense staining in invasive ovarian cancers and metastases. Microinvasive cells in LMP tumors with disruption of the basement membrane stained strongly positive for MMP-2. In contrast, an immunohistochemical study by Westerlund *et al.* (15) showed no statistical difference between benign lesions, LMP tumors, and epithelial ovarian cancers. To get additional information on MMP-2 in ovarian cancer, we used gelatin zymography and found no difference in pro-MMP-2 activity between benign and malignant tissues. In contrast, active MMP-2 was detected in cancer tissue but never in benign or LMP tumors. Total MMP-2 activity (pro-MMP-2 + MMP-2) and the ratio of



**Fig. 5** Box plot. Antigen concentrations of (A) uPA and (B) PAI-1 in tissue extracts of ovarian serous cystadenomas, LMP tumors, and primary tumors and corresponding omentum metastases of advanced ovarian cancer FIGO IIIc/IV. The *bottom* boundary of the box plot represents the value of the 25<sup>th</sup> percentile; the *top* boundary represents the value of the 75<sup>th</sup> percentile. The *bars* indicate minimum and maximum values without extremes. Median values are marked within the boxes by *bold horizontal bars*. Statistically significant differences between groups are indicated by *Ps*.

activated MMP-2:pro-MMP-2 was higher in metastases when compared with the primary tumor. These findings are in agreement with studies in breast cancer (22) and gastric cancer (29) where in the tumors, the active form of MMP-2 was found significantly elevated compared with the latent form. This suggests that in ovarian cancer, tumor cells with MMP-2 activity are more probable to metastasize or, alternatively, that ovarian tumor cells produce more active MMP-2 after metastasis. Two factors could be responsible for the activation of pro-MMP-2 in ovarian cancer, either low levels of the inhibitor tissue inhibitor of metalloproteinase-2, which binds to pro-MMP-2, or high expression of MT1-MMP (MMP-14), a physiological activator of pro-MMP-2. Indeed, expression of MT1-MMP was detected in ovarian cancers but never in benign tumors (11). These and our findings suggest that conversion of pro-MMP-2 to active MMP-2 is an important step in the transition from a benign to a malignant ovarian tumor.

Analogous to the gradual increase in MMP-2, we noted a correlation between pro-MMP-9 protein expression and the malignant potential of the ovarian tumor with the highest median pro-MMP-9 concentration in metastases of ovarian cancer. Thus far, only a few data on MMP-9 expression in ovarian cancer are available. Kikkawa *et al.* (30) in 1997 reported that the ratio of MMP-9:MMP-2 was elevated in ovarian cancer compared with benign ovarian tumor tissue. In colon cancer, immunohistochemical staining of MMP-9 was negative in normal mucosa and adenoma, whereas it was detected in 69% of primary tumors and in all of the 10 liver metastases investigated (31). In gastric cancer, MMP-9 activity as measured by zymography was lower in the normal mucosa and early gastric cancer compared with advanced cancer (32). These data are in accordance with our findings of elevated MMP-9 expression in malignant ovarian tissue and indicate that pro-MMP-9 expression is an important marker for progression from benign lesions to ovarian cancer. Interestingly, we did not detect the active form of MMP-9 (82 kDa) in either benign or malignant ovarian tissues. This is in agreement with earlier zymographic analyses of 29 ovarian cancer tissues by Naylor *et al.* (12), who also detected only pro-MMP-9 (92 kDa). However, pro-MMP-9 in ovarian tissue can be activated *in vitro*, as evidenced by our experiments activating pro-MMP-9 by APMA. The increase in pro-MMP-9 in ovarian cancer and their corresponding metastases could be attributable to the infiltration of inflammatory cells that express a high amount of pro-MMP-9 and often surround malignant tumors (33). The lack of activated MMP-9 might help to explain the peculiar tumor biology of epithelial ovarian cancers, which rarely metastasize outside the peritoneal cavity (34). Interestingly, in colon cancer (32) and breast cancer (22) that often show distant metastases the activated form of MMP-9 was detected in the tumor tissue, suggesting indirectly a role for active MMP-9 in distant metastases.

The importance of uPA and its inhibitor PAI-1 in ovarian cancer has been shown by us and others (16–19). In patients with advanced ovarian cancer stage FIGO IIIc, PAI-1 turned out to be a strong, statistically independent prognostic parameter only surpassed by residual tumor mass and was more important than the clinical parameters nodal status, ascites volume, grading, and age (17, 35). We extend these findings now by showing that antigen levels of uPA and PAI-1 were both low in benign tumors but increased significantly from LMP tumors to advanced ovarian cancer. Levels were even higher in the primary tumors than in the corresponding metastases.

Our finding of a low correlation between uPA and MMP-2/MMP-9 expression in ovarian tumors was surprising because *in vitro* data suggested a common regulatory mechanism and a mutual regulation. An invasion assay performed by Ellerbroek *et al.* (36) indicated that uPA as well as MMP-9 are associated with ovarian cancer cell invasion. In addition, uPA and plasmin binding to the tumor cell surface is involved in activation of MMP-2 (37); therefore, coexpression of MMPs and uPA *in vivo* would have been an expected finding. However, we found only a weak correlation between uPA and MMP-2/MMP-9 expression in ovarian tumors. One explanation might be the different transcriptional regulation of uPA, MMP-2, and MMP-9, which is overlapping but not identical (38–40). The weak correlation of MMP-2 and uPA also implies that in ovarian cancer other

proteins besides uPA/plasmin are involved in the conversion of pro-MMP-2 to its active form.

In summary, our results on elevated expression of uPA, PAI-1, MMP-9, and MMP-2 in ovarian cancer endorse the significance of these proteolytic factors in tumor invasion and metastasis in ovarian cancer. From our data, inhibition of MMP-2 activation would be an interesting approach toward a biological therapy of ovarian cancer, because MMP-2 was never converted to its active form in benign ovarian tumors or LMP, but activated in 66% of advanced ovarian cancers and in 93% of the corresponding metastases.

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