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Cytokine-regulated expression of collagenase-2 (MMP-8) is involved in the progression of ovarian cancer

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Abstract

Matrix metalloproteinases (MMPs) have been implicated in ovarian cancer progression. Among them, MMP-8 that degrades type I collagen may play a crucial role. The aim of our study was to determine MMP-8 expression and regulation in ovarian cancer and its association with other MMPs and tissue inhibitors of metalloproteinases (TIMPs). Tissue microarrays (TMAs) containing tissue cylinders from 302 patients were used for immunohistochemical studies. In addition, MMP-8 expression *in vitro* was analysed by a specific immunoassay and PCR-analysis. MMP-7 (81%), MMP-8 (95%), MT3-MMP (100%), TIMP-2 (100%), and TIMP-3 (96%) were expressed in all the OVCAs, but the staining intensities varied. MMP-3 (6%), MMP-9 (57%) and TIMP-1 (43%) expressions were more rarely detected. Only MMP-8 expression levels correlated with tumour grade ($P<0.01$), tumour stage ($P<0.01$), and a poor prognosis ($P<0.05$). MMP-8 protein and gene expression *in vitro* was found to be significantly upregulated by interleukin-1beta (IL-1β, $P<0.01$). The data indicate that MMP-8 overexpression in OVCAs is regulated by IL-1β and that pro-inflammatory cytokines may promote the invasive potential of ovarian cancer.

Keywords: Matrix metalloproteinases; Collagenase-2; MMP-8; Ovarian cancer; Pro-inflammatory cytokines; Tissue inhibitor of matrix metalloproteinases; TIMP; Tissue microarray; TMA

1. Introduction

Ovarian cancer is the leading cause of death from gynaecological malignancy in Western countries [1]. Invasive growth and metastasis of ovarian cancer is a multistep process involving degradation of the basement membrane and proteolysis of the extracellular matrix (ECM) [2]. Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent degrading enzymes, have been implicated in ovarian cancer progression [3–5]. Recent studies have demonstrated that ovarian carcinomas (OVCAs) *in vivo* and *in vitro* produce a variety of MMPs that are capable of cleaving basement membrane constitutes (MMP-2, MMP-7, MMP-9), as well as ECM components (MMP-1) at the tumour–host interface [3,4,6–13]. In addition, OVCAs have been demonstrated to express MT1-MMP and MT2-MMP, two matrix metalloproteinases that have been implicated in both degradation of basement membrane components and activation of soluble MMPs [3,7,12].

Among the different MMPs, collagenase-2 (MMP-8) may play a crucial role in the invasion process of ovarian cancer. MMP-8 is the most active proteinase against type I collagen, the major structural component of the ECM [14]. It is expressed in various benign and malignant cells including chondrocytes, fibroblasts, inflammatory, endothelial and squamous carcinoma cells [15–18]. The activity of MMP-8 is regulated by specific tissue inhibitors of metalloproteinase (TIMPs) and by interleukin-1beta (IL-1β) and tumour

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necrosis factor-alpha (TNF-α) [15,17,19,20]. High levels of these pro-inflammatory cytokines can be detected in the serum and ascitic fluid of patients with ovarian cancer [21,22] yielding an increased secretion of cytokines and growth factors by the tumour cells [23,24]. Recent studies on benign cells have demonstrated a significant induction of MMP-8 by IL-1β and TNF-α [15,17,20]. However, data regarding expression of MMP-8 and its regulation by pro-inflammatory cytokines in ovarian cancer cells are still lacking.

Therefore, the aims of the study were to (1) investigate MMP-8 expression and regulation in OVCAs, (2) demonstrate its association with other MMPs and TIMPs, and (3) evaluate the prognostic significance of MMPs expression.

2. Patients and methods

2.1. Patients

Histological typing, grading, and tumour staging of 302 OVCAs examined at the Institute of Pathology, Kantonsspital Basel (Switzerland) was done by one pathologist. Tumours were graded according to Silverberg criteria [25]. Tumour staging was established according to International Federation of Gynecology and Obstetrics (FIGO) criteria [26]. Patient age ranged from 24 to 84 years, (median 60 years). There were 119 serous, 40 mucinous, 68 endometrioid, 16 undifferentiated, 16 mullerian, 24 clear cell carcinomas, 5 malignant Brenner, 10 sex cord-stromal, and 4 yolk sac tumours. 87 were grade I, 88 grade II and 94 grade III tumours. 58 cases were staged as FIGO I, 30 cases as FIGO II, 132 cases as FIGO III and 13 cases as FIGO IV. Tumour grade and stage could not be determined for 33 and 69 patients due to missing clinical data. Follow-up period ranged from 0.5 to 210 months (median 43 months). Residual tumour was defined as tumour masses $\leqslant 2$ cm^2 or >2 cm^2 remaining in the peritoneal cavity after surgical treatment. In 35 of 123 patients, residual tumour remained after surgery (19 patients $\leqslant 2$ cm^2, 16 patients >2 cm^2). Overall and tumour-specific survival data were obtained from the Cancer Registry Basel (Dr G. Jundt) and from communication with the clinicans.

2.2. Tissue microarray (TMA)

Tissue samples were fixed in buffered 4% formalin, embedded in paraffin, and used to construct a TMA (Fig. 1) [27–29]. Haematoxylin-eosin stained sections were made from each selected primary tumour block (donor blocks) to define representative tumour regions. Tissue cylinders (0.6 mm in diameter) were punched from the region of the donor block with the use of a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA) and transferred to a 25 mm×35 mm paraffin block. The resulting TMA block was cut into 3 μm sections and transferred to glass slides by use of the Paraffin Sectioning Aid System (Instrumedics, Hackensack, NJ, USA). Separate sections from the TMA block were used for immunohistochemical analysis (Fig. 1b–d).

2.3. Immunohistochemistry

Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite, Vectra Laboratories) [28,29]. Monoclonal antibodies against MMP-3,-7,-8,-9, MT3-MMP, TIMP-1,-2, and-3 were used as primary antibodies (Table 1). TMA was analysed by light microscopy by use of a 10× objective. Immunostaining results were scored semiquantitatively by one pathologist with a weighted score. The percentage of the total number of positive tumour cells was categorised and awarded a score of 0 to 2: $0<5\%$; 1: 5–29%; 2: $\geqslant 30\%$. The intensity of staining was scored on

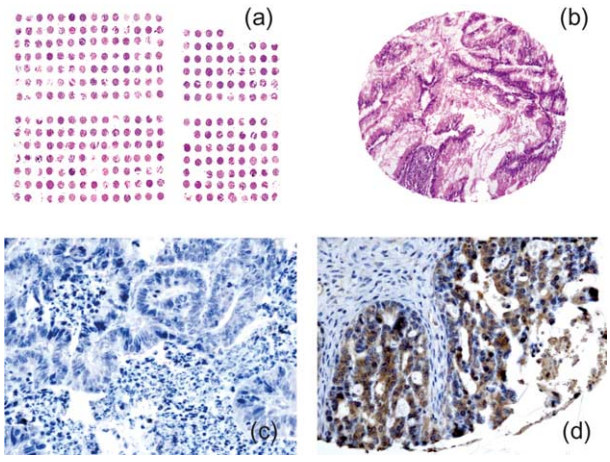

Fig. 1. (a) Ovarian cancer tissue microarray containing representative samples of 302 randomly selected patients with OVCA. (b) Detail of the tissue microarray showing a mucinous ovarian carcinoma (H&E staining, original magnification 20×). Separate sections from the tissue microarray were used for immunohistochemical detection of MMP-8 (Original magnifications 200×): (c) No expression of MMP-8 (endometrioid ovarian carcinoma), (d) Strong expression of MMP-8 (serous ovarian carcinoma).

Table 1
Antibodies and dilutions used for immunohistochemistry

Antigen	Host	Antibody	Dilution	Source
MMP-3	Mouse	Monoclonal	1:40	Novocostra
MMP-7	Mouse	Monoclonal	1:100	Calbiochem
MMP-8	Mouse	Monoclonal	1:20	Oncogene
MMP-9	Mouse	Monoclonal	1:160	Novocostra
MT3-MMP	Mouse	Monoclonal	1:80	Oncogene
TIMP-1	Mouse	Monoclonal	1:80	Oncogene
TIMP-2	Mouse	Monoclonal	1:480	Oncogene
TIMP-3	Mouse	Monoclonal	1:200	Oncogene

a 3-point scale: 1, weak; 2, moderate; 3, intense. A weighted score for each tumour specimen was produced by multiplying the percentage score with the intensity score. Then three groups of the weighted score were categorised: −, no expression (weighted score 0); +, weak expression (weighted scores 1, 2, 3); ++, strong expression (weighted scores 4, 6).

2.4. Modulation of MMP-8 expression

The established human OVCA cell lines HTB-77, 2780 and OVCAR-3 (kindly provided by Dr T. Hamilton, NIH, Bethesda, Dr G. Gastl, Department of Medicine, University of Innsbruck and Dr C. Dittrich, Vienna, Austria) were cultured in 25 cm^2 culture flasks (Greiner, Kremsmuenster, Austria) in Roswell Park Memorial Institute (RPMI)-1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, penicillin/streptomycin (200 IU/ml, 100 μg/ml; Biochrom), and grown to confluence at 37 °C in a humidified atmosphere of 5% CO_2 in air.

For determination of *MMP-8* mRNA expression, OVCA cells were detached with trypsin/versene (0.05%/0.02%), washed once in medium and $37.5–45\times10^3$ cells seeded into 6-well tissue culture plates (9.6 cm^2/well) in a volume of 5 ml/well. After 3 days, exponentially growing cells were incubated for 3, 6, 12 and 24 h in the absence or presence of 1 ng/ml IL-1β (R&D Systems Biomedica, Vienna, Austria) or TNF-α Sigma Vienna, Austria). At the end of the incubation period, cells were detached, centrifuged and the pellet stored at −70 °C until used in the reverse transcription-polymerase chain reaction (RT-PCR) analysis.

For determination of MMP-8 protein secretion, OVCA $2.5–6\times10^3$ cells were detached as described above with trypsin/versene (0.05%/0.02%), washed once in medium and seeded into 24-well tissue culture dishes in a volume of 1 ml culture medium. After 3 days, cells were incubated for 48 h in the absence or presence of the appropriate cytokines (0.01–0.1–1–10 ng/ml). Then supernatants were removed and stored at −70 °C until analysis. Cell number was determined using a Cell Analyzer System (CASY, Schärfe System, Reutlingen, Germany).

2.5. Determination of MMP-8 mRNA expression by PCR analysis

PCR was performed by using primer pairs described below with the following conditions: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Control PCRs lacking cDNA were performed for each set of reactions. PCR reaction products were loaded onto 2.5% agarose gels, stained with ethidium bromide, and visualised under ultraviolet (UV) illumination. The *MMP-8* primer sequence was designed based on the published mRNA sequence as follows: 5′-TCA AGC AAC CCT ATC CAA CC-3′ (sense) and 5′-CTT GCT GGA AAA CTG CAT CA-3′ (antisense).

2.6. Determination of MMP-8 protein secretion by enzyme-linked immunosorbent assay (ELISA)

MMP-8 secretion in cell culture supernatants was assayed using specific sandwich enzyme immunoassays (ELISA) (Quantikine DMP800, R&D Systems, Wiesbaden-Nordenstadt, Germany). 50 μl of the supernatants were incubated in wells of a microplate precoated with a monoclonal antibody specific for MMP-8. After washing, an enzyme-linked specific monoclonal MMP-8 antibody was added. Following a wash to remove any unbound antibody-enzyme reagent, samples were incubated in a substrate solution. After 2 h, the colour development was stopped and intensity of the colour measured by spectrophotometry. Data from three independent experiments were expressed as picograms per 10^6 cells.

2.7. Data analysis

Comparisons of the rates of MMPs and TIMPs expression with histopathological variables, including tumour grade, tumour stage, and histologic type were performed using Spearman's rank-order correlation. Analysis of MMPs and TIMPs expression with respect to disease status and other MMPs or TIMPs was restricted to serous, mucinous, endometrioid and undifferentiated ovarian carcinomas due to their comparable biological behaviour. A P-value <0.05 was considered to be statistically significant. Comparison with survival was performed using a Kaplan–Meier survival analysis. Overall survival was defined as the time between tumorectomy and patient death. Statistical differences between the groups were determined with the log rank test. A Cox proportional hazard analysis was used to test for independent prognostic parameters. Statistical analysis of *in vitro* studies was performed using the two-tailed Student's t test. Experiments were run in duplicate. All data *in vitro* are presented as means ± standard errors of the means (SEM).

3. Results

3.1. In vivo expression of MMPs and TIMPs in ovarian cancer

Ninety-five percent of OVCAs showed positive cytoplasmic MMP-8 expression with little variation between the different tumour subtypes (Table 2). Importantly, major variations of the MMP-8 staining intensity were

Table 2
MMPs expression *in situ* in ovarian carcinomas

Tumour type		Expression level (%)[a]														
		MMP-3			MMP-7			MMP-8			MMP-9			MT3-MMP		
	n	–	+	++	–	+	++	–	+	++	–	+	++	–	+	++
Serous	119	97	3	0	9	21	70	0	6	94	36	47	17	0	3	97
Mucinous	40	97	3	0	27	15	58	18	18	64	49	43	9	0	0	100
Endometrioid	68	97	3	0	25	14	61	7	12	81	51	46	3	0	5	95
Undifferentiated	16	93	7	0	7	27	67	7	7	86	43	43	14	0	8	92
Mullerian	16	92	8	0	33	33	33	0	0	100	36	45	18	0	15	85
Clear cell	24	59	41	0	17	39	43	0	17	83	39	52	9	0	16	84
Malignant brenner	5	100	0	0	50	25	25	0	0	100	75	0	25	0	33	67
Sex cord-stromal	10	100	0	0	38	25	38	0	0	100	50	38	13	0	29	71
Yolk sac	4	100	0	0	25	50	25	0	0	100	50	50	0	0	25	75

[a] The expression level was scored as –, no expression; +, weak expression; ++, strong expression (for definition see text in Patients and Methods).

observed (Fig. 1b–d). Absent or only weak expression was noted in 36% of mucinous and 19% of endometrioid OVCAs. MMP-8 expression levels proved to be significantly associated with tumour grade ($P<0.01$) and tumour stage ($P<0.01$). An intense expression was found in 75% of grade I, 86% of grade II, 92% of grade III, in 77% and 74% of FIGO stage I and II, and in 93% and 91% of FIGO stage III and IV tumours, respectively (Table 3). Additionally, MMP-8 expression was associated with MMP-9 ($P<0.01$), TIMP-2 ($P<0.001$) and TIMP-3 ($P<0.001$) expression. MMP-8 expression significantly correlated with overall survival ($P<0.05$, Fig. 2). Data on patient survival was available for 107 cases. There were no cases without MMP-8 expression included in the survival analysis due to a lack of survival information in these 32 tumours. Within the category of non-organ-confined tumours (FIGO stages II–IV), survival was significantly better for OVCAs with weak or negative MMP-8 expression than those with intense MMP-8 expression ($P<0.05$). However, in a multivariate analysis including the variables tumour stage, MMP-8 expression, presence of residual tumour, tumour grade and tumour histotype, MMP-8 expression did just not reach the significance level ($P=0.05$) for an independent prognostic factor (Table 4).

Cytoplasmic immunostaining for MMP-7 and MT3-MMP was detected in the majority of the OVCA subtypes, whereas MMP-3 and MMP-9 were rarely expressed (Table 2). The degree of differentiation or the tumour stage were not associated with MMP-7, MMP-9 or MT3-MMP expression. MMP-3 negatively correlated with FIGO tumour stage ($P<0.05$; Table 3). MMP-3 ($P<0.05$) and MMP-7 ($P<0.001$) expression was associated with MMP-9 expression. MMP-7 expression correlated with MT3-MMP ($P<0.01$), TIMP-1 ($P<0.01$), TIMP-2 ($P<0.05$), and TIMP-3 ($P<0.01$) expression. MMP-9 was associated with MT3-MMP ($P<0.05$), TIMP-1 ($P<0.001$), TIMP-2 ($P<0.05$) and TIMP-3 ($P<0.01$) expression. MMP-3, MMP-7, MMP-9, MT3-MMP expressions were not

Table 3
Association between MMPs expression *in situ*, differentiation grade, and tumour stage in ovarian carcinomas

Parameters		Expression level (%)[a]														
		MMP-3			MMP-7			MMP-8			MMP-9			MT3-MMP		
	n	–	+	++	–	+	++	–	+	++	–	+	++	–	+	++
Tumour grade																
Grade I	87	100	0	0	20	16	64	10	15	75	48	44	8	0	3	97
Grade II	88	94	6	0	14	16	70	6	8	86	34	52	13	0	3	97
Grade III	94	96	4	0	16	22	62	1	7	92	45	43	12	0	4	96
Tumour stage																
FIGO I	58	92	8	0	24	9	67	11	11	77	54	40	6	0	0	100
FIGO II	30	100	0	0	20	20	60	11	16	74	57	38	5	0	6	94
FIGO III	132	99	1	0	17	22	61	1	5	93	37	49	14	0	5	95
FIGO IV	13	100	0	0	0	9	91	9	0	91	55	27	18	0	0	100

[a] The expression level was scored as –, no expression; +, weak expression; ++, strong expression (for definition see text in Patients and Methods).

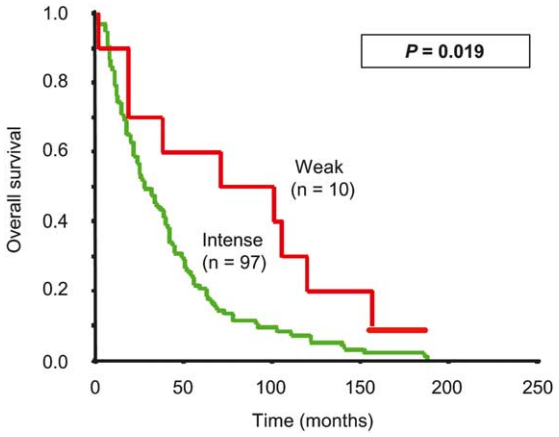

Fig. 2. Kaplan–Meier survival curves demonstrating the association between MMP-8 expression in FIGO stage I–IV ovarian cancers ($n=107$) and disease outcome ($P<0.05$).

Table 4
Proportional hazards analysis in ovarian carcinoma

Variable	*P* value
Tumour stage (FIGO)	<0.001
MMP-8 expression	0.05
Presence of residual tumour	0.12
Tumour grade	0.13
Histotype	0.22

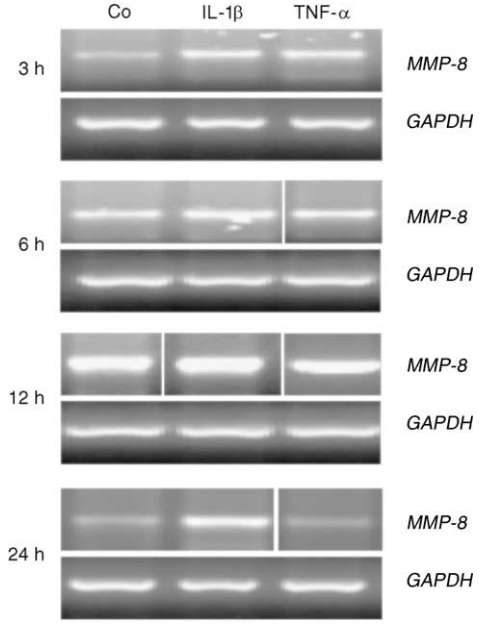

Fig. 3. Detection of *MMP-8*-specific transcripts by PCR analysis. Total RNA of the ovarian cancer cell line 2780 was isolated from untreated ovarian cancer cells or from ovarian cancer cells treated for 3-6-12-24 h with IL-1β or TNF-α (1 ng/ml each). PCR reactions from three independent experiments were performed in duplicate. Co, control.

associated with patient outcome. Only when all ovarian cancer subtypes were included in the survival analysis MMP-9 was significantly correlated with tumour stage and tumour-specific survival ($P<0.05$).

Strong TIMP-2 and TIMP-3 expression was seen in most of the ovarian cancer subtypes, whereas TIMP-1 expression was only rarely found. All of the investigated TIMPs were significantly correlated with tumour grade (TIMP-1, $P<0.01$; TIMP-2, $P<0.05$; TIMP-3, $P<0.001$), but only TIMP-3 ($P<0.01$) expression was related to tumour stage. TIMP-1 and TIMP-2 expression were associated with positive TIMP-3 staining ($P<0.05$). No significant correlation was found between TIMPs expression and patient survival.

3.2. In vitro *expression of MMP-8 in ovarian carcinomas*

One of the three OVCA cell lines (2780) expressed *MMP-8* mRNA (Fig. 3). The expression of *MMP-8*-specific transcripts of 502 base pairs was demonstrable in controls as well as in IL-1β- or TNF-α-treated OVCA cells. Fig. 4 shows the expression of MMP-8 protein in control and cytokine-stimulated cells of the 2780 cell line. Mean levels of 34.0 ± 11.7 pg/10^6 cells were achieved within 48 h. Mean MMP-8 levels induced by IL-1β were 190.2 ± 32.0 pg/10^6 cells, 5.6-fold ($P<0.01$) over baseline values. Treatment of 2780 with TNF-α

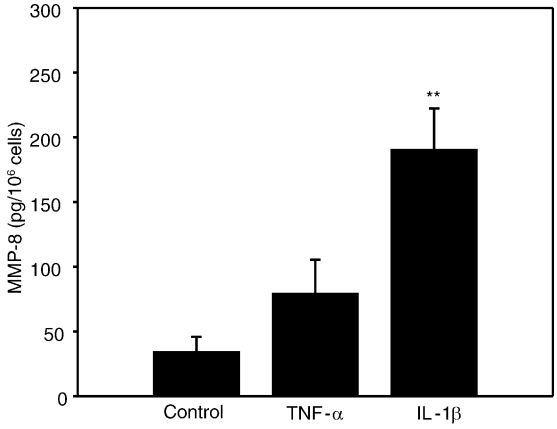

Fig. 4. Secretion of MMP-8 by the ovarian carcinoma cell line 2780. Cells were treated for 48 h with the pro-inflammatory cytokines IL-1β or TNF-α (1 ng/ml each). MMP-8 protein was measured in duplicate by ELISA in the supernatants of the ovarian carcinoma cell cultures. Results are the means (±SEM) of MMP-8 release from three independent experiments expressed as pg/10^6 cells. $^{**}P<0.01$.

resulted in a 2.3-fold (79.0 ± 26.5 pg/10^6 cells) increase of MMP-8 secretion, but this induction was non-significant ($P=0.15$). The induction of MMP-8 secretion by IL-1β was dose-dependent. An 1.4-fold induction was already achieved with an IL-1β dose of 0.1 ng/ml ($P=0.40$) with a maximum induction being observed at doses of 1 ng/ml.

4. Discussion

The present study represents a comprehensive analysis of MMP-3, MMP-7, MMP-8, MMP-9, and MT3-MMP expression in OVCAs. Our data confirm expression of MMP-7 and MMP-9 by ovarian cancer cells and provide new data on MMP-3, MMP-8 and MT3-MMP expression. Weak MMP-3 and intense staining of MT3-MMP was frequent in OVCAs. However, both MMP subtypes showed no significant association with differentiation grade. MMP-3 expression was significantly associated with advanced tumour stage of ovarian cancer. In contrast, the vast majority of ovarian cancer subtypes displayed a very strong MMP-8 expression which significantly correlated with tumour grade and tumour stage suggesting MMP-8 is an important factor for ovarian cancer progression. Strong MMP-8 expression was seen in association with intense TIMP-2 and TIMP-3 expression indicating that MMP-8 may be predominantly regulated by these two metalloproteinase inhibitors. MMP-8 overexpression in OVCAs was also associated with MMP-9 immunostaining suggesting that degradation of type IV collagen (MMP-9) [30] of the basement membrane in addition to cleaving of type I collagen (MMP-8) [2] at the tumour–host interface may be an important step in ovarian cancer cell invasion.

The expression of MMP-9 in OVCAs has been confirmed by several studies [3,6,7,9,10,12]. It has been shown that mRNA levels of MMP-9 correlate with tumour progression and poor survival in ovarian cancer [3,7,10]. In our study, we could not confirm MMP-9 as prognostic parameter for ovarian cancer when the analysis was restricted to the more common ovarian carcinoma subtypes. However, when all OVCA subtypes were included in a survival analysis, MMP-9 correlated with tumour stage and poor outcome. In addition, MMP-9 overexpression was strongly associated with reduced immunostaining for TIMP-1. These findings support the hypothesis that MMP-9 overexpression and downregulation of TIMP-1 may play a certain role in ovarian cancer invasion [3,7,9]. In the present study, the *in vivo* production of MMP-8 by ovarian cancer cells was also demonstrated by *in vitro* studies, where MMP-8 expression was observed in one OVCA cell line. MMP-8 production was markedly upregulated in response to IL-1β, a cytokine that has been shown to participate in the regulation of disease processes of the peritoneal cavity [23,24]. *MMP-8* mRNA expression was evident already after 3 h of culture suggesting that upregulation of MMP-8 may constitute an early event in inflammatory processes. These findings are consistant with recent studies on human fibroblasts demonstrating that treatment of cells with IL-1β increases *MMP-8* gene transcription within 6 h [20]. Moreover, it has been shown that MMP-8 expression is enhanced during inflammatory conditions indicating that pro-inflammatory cytokines, particularly IL-1β plays a central role in the modulation of MMP-8 expression [31].

In summary, our data demonstrate MMP-3, MMP-7, MMP-8, MMP-9, MT3-MMP, and TIMPs-1,-2, and-3 expression in ovarian cancer cells and identify MMP-8 as a prognostic factor for ovarian cancer progression. In addition, we have shown that MMP-8 expression is upregulated by IL-1β, suggesting that cytokine-associated overexpression of MMP-8 by OVCAs may facilitate the invasive behaviour of ovarian cancer.

5. Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence the present work.

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