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Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-α dependent up-regulation of matrix metalloproteases

Thorsten Hagemann[1], Stephen C.Robinson[2], Matthias Schulz[1], Lorenz Trümper[1], Frances R.Balkwill[3] and Claudia Binder[1,4]

[1]Department of Haematology/Oncology, Georg-August-University, Göttingen, Germany, [2]Cancer Research Institute, Departments of Laboratory Medicine, Pathology, and Comprehensive Cancer Center, University of California, San Francisco, USA and [3]Cancer Research UK Translational Oncology Laboratory, Barts and The London, Queen Mary's School of Medicine and Dentistry, Charterhouse Square, London, UK

[4]To whom correspondence should be addressed
Email: cbinder@med.uni-goettingen.de

Apart from the neoplastic cells, malignant tumours consist of the extracellular matrix (ECM) and normal cells, in particular tumour-associated macrophages (TAM). To understand the mechanisms by which TAM can influence tumour cell invasion we co-cultured the human breast cancer cell lines MCF-7, SK-BR-3 and the benign mammary epithelial cell line hTERT-HME1 with macrophages. Co-incubation enhanced invasiveness of the tumour cells, while hTERT-HME1 remained non-invasive. Addition of the broad-spectrum matrix metalloprotease (MMP)-inhibitor FN 439, neutralizing MMP-9 or tumour necrosis factor-alpha (TNF-α) antibodies reduced invasiveness to basal levels. As shown by zymography, all cell lines produced low amounts of MMP-2, -3, -7 and -9 under control conditions. Basal MMP production by macrophages was significantly higher. Upon co-incubation, supernatant levels of MMPs -2, -3, -7 and -9 increased significantly, paralleled by an increase of MMP-2 activation. MMP-2 and -9 induction could be blocked by TNF-α antibodies. Co-culture of macrophages and hTERT-HME1 did not lead to MMP induction. In the co-cultures, mRNAs for MMPs and TNF-α were significantly up-regulated in macrophages, while the mRNA concentrations in the tumour cells remained unchanged. In summary, we have found that co-cultivation of tumour cells with macrophages leads to enhanced invasiveness of the malignant cells due to TNF-α dependent MMP induction in the macrophages.

Introduction

Malignant tumours consist of both neoplastic cells and non-neoplastic cellular elements including fibroblasts, endothelial cells, lymphocytes and macrophages surrounded by an extracellular matrix (ECM) (1). Interactions between neoplastic cells and the surrounding microenvironment are crucial to each step of tumourigenesis. There is evidence that stromal fibroblasts interfere with proliferation, differentiation and invasion of cancer cells at both primary and metastatic sites (2,3).

Abbreviations: ECM, extracellular matrix; MMP, matrix metalloprotease; TAM, tumour-associated macrophages; TNF-α, tumour necrosis factor-alpha.

Tumour-associated macrophages (TAM) represent a major component of the lymphoreticular infiltrates of tumours (4). High numbers of TAM have been observed in many tumours, such as invasive breast cancers, where the neoplastic cell population may even be outnumbered by stromal cells (5). Although activated macrophages may have antitumour activity, the extent of the macrophage infiltrate correlates positively with angiogenesis and negatively with prognosis in some cancers (6,7). There is growing evidence that tumour-derived molecules, e.g. tumour necrosis factor-alpha (TNF-α), epidermal growth factor, vascular endothelial growth factor redirect TAM activities to promote tumour survival and growth (8–13).

A crucial step for invasion and metastasis is the destruction of biological barriers such as the basement membrane, and this requires activation of proteolytic enzymes (14,15). Matrix metalloproteases (MMP), which belong to a large family of 24 highly homologous, zinc-dependent, ECM-degrading proteases, play an essential role in this context (16). They play an important role in tumour angiogenesis, metastasis and growth factor release from the ECM (14,17). Several MMPs, including MMP-9 (18), are directly involved in cancer invasion (19,20). In clinically invasive breast cancers, augmented protein levels and activity of MMP-9 and -2 were associated with shortened survival and unfavourable prognosis (21).

MMP production and activation is dependent on various cytokines, among others TNF-α (8,9,13). MMPs are produced by diverse tumour components. Tumour cells, as well as fibroblasts in the surrounding stromal compartment, have been shown to express MMPs (22–24). In addition, TAMs are able to produce a variety of MMPs including MMP-1, -2, -7, -9 and -12 (25). However, it is not clear whether interactions between tumour cells and macrophages contribute to MMP up-regulation and tumour cell invasion.

To address this question, two human breast cancer cell lines (MCF-7, SK-BR-3) and the immortalized benign mammary epithelial cell line hTERT-HME1 were co-cultured with macrophages, which had been derived from human peripheral blood monocytes. Co-culture induced MMP expression and activity predominantly in the macrophages and resulted in enhanced invasiveness of the malignant, but not the benign, cell lines.

Materials and methods

Cells lines and reagents

If not indicated otherwise, all substances were purchased from Sigma (Deisenhofen, Germany). The MCF-7 and SK-BR-3 human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD), the telomerase-immortalized benign human breast epithelial cell line hTERT-HME1 from BD Biosciences Clontech (Heidelberg, Germany). The cell lines were grown on RPMI-1640 medium supplemented with 10% fetal bovine serum, which had been treated with gelatin immobilized on cross-linked 4% beaded-agarose as described before (25). The serum was then tested by zymography to contain no significant amounts of gelatinases and caseinases. All experiments were performed under endotoxin-free conditions.

Microinvasion assay

Invasion was measured by assessment of the breast cancer cell migration rate through an artificial basement membrane in a modified Boyden chamber, where the various cellular components were grown without direct cell-to-cell contact. The membrane consisted of polycarbonate (10 μm pore diameter, Nucleopore®, Pleasanton, CA) and was coated on ice with Matrigel (ECM gel) diluted 1:3 in serum-free RPMI. MCF-7, SK-BR-3 or hTERT-HME1 cells (2×10^5 cells/ml RPMI) were seeded into the upper well of the chamber, while the lower well was filled up to the top with RPMI + 10% FCS as a chemoattractant. In the experiments, where MMP activity was inhibited, the non-selective MMP inhibitor FN 439 (MMP inhibitor I, Calbiochem-Novabiochem, Bad Soden, Germany) was added at various concentrations. The neutralizing TNF-α antibody 28401.111 (R&D, Abingdon, UK) or the neutralizing MMP-9 antibody 6-6B (Calbiochem-Novabiochem, Boston, MA) was added after plating the cells and renewed every 24 h during the incubation period.

Human macrophages were derived from peripheral blood mononuclear cells. After centrifugation over a Ficoll-Hypaque density gradient, mononuclear cells were plated in RPMI-1640 + 10% FCS. Monocytes were allowed to adhere and cultivated until differentiation into macrophages as assessed by morphologic and functional criteria. For co-culture experiments 2×10^5 macrophages/ml RPMI were seeded in transwell inserts (Nunc, Wiesbaden, Germany), the bottom of which consists of a membrane permeable for liquids but not for cells. The transwells were inserted into the upper well of the Boyden chamber. To activate macrophages, bacterial lipopolysaccharide (*Escherichi coli* 026:B6 LPS) was used at 10 μg/ml.

After 96 h (or the indicated time panel), the content of the lower well with floating as well as adherent cells was removed and pelleted by centrifugation. The supernatant was used for zymography (see below). The cell pellet was resolved in 200 μl PBS and spun down on 12 mm cover slips. After air-drying, the cytospins were stained with 4′,6′-diamino-2′-phenylindol (DAPI, 200 ng/ml). Cover slips were mounted in 20% mowiol 4-88 (Hoechst, Frankfurt, Germany) on glass slides. Intact nuclei were counted by UV-microscopy (Axioskop, Zeiss, Germany) and documented with a digital image editing system (Adobe Photoshop 3.0, Adobe Systems, Tokyo, Japan). To assess the morphology of the migrated cells, control stainings with haematoxylin and eosin were performed, which yielded the same results. All experiments were performed at least in triplicate.

RNA extraction and quantitative RT–PCR

Total RNA was extracted from MCF-7, SK-BR-3, hTERT-HME1 and macrophages with the guanidinium thiocyanate method (26). Reverse transcription was performed from 2 μg of total RNA using oligo-dT primers and M-MLV reverse transcriptase (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for MMP-3, -7, -8, -9, -10 and -13 were designed as described before (27,28) and synthesized by Biometra (Göttingen, Germany). Primers for MMP-1, -2, -11, -12 and -14 were synthesized according to Giambernardi *et al.* (29).

Quantitative PCRs were performed on the Light Cycler PCR Analysis System® (Roche Diagnostics). The amount of generated DNA was measured by fluorescence detection of the ds-specific DNA-binding dye SYBR Green I (Roche Diagnostics) and quantified in relation to serial dilutions of a known standard. The detailed procedure for quantification has been described previously (28). The PCR reaction contained a standard PCR buffer, SYBR Green I (1:20 000), bovine serum albumin (0.05%) and 5 pmol of the specific sense and antisense primers. Forty cycles were performed, 0 s denaturation at 94°C, 5 s annealing at the respective optimal annealing temperature, 10 s extension at 72°C and 5 s fluorescence detection at 72–84°C. The melting curves were obtained at the end of amplification by cooling the sample at 20°C/s to 72°C and then increasing the temperature to 95°C at 0.1°C/s. Fluorescence was acquired every 0.1°C. To confirm comparable efficiency of reverse transcription in the samples, β-actin was amplified and quantified according to the same protocol (primers from BD Biosciences Clontech). Experiments were performed at least in triplicate. TNF-α multiplex real-time PCR analyses was performed as described before (25) using pre-made TNF-α (FAM) and 18 s rRNA (VIC)-specific primers and probes with the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Warrington, UK). TNF-α PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 5 μl cDNA in a 25-μl final reaction mixture. The cycling conditions were incubated at 50°C for 2 min, followed by 10 min at 95°C and 40 cycles of 15 s at 95°C, and 1 min at 60°C. Experiments were performed in triplicate for each sample. TNF-α was normalized to the 18S RNA, and fold difference calculated as described before (25).

Enzyme-linked immunoabsorbent assays

The supernatant was harvested under sterile conditions, frozen and stored at −20°C. TNF-α concentration was determined using a commercial ELISA kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Zymography

Cells incubated as indicated and the respective supernatants were used for the experiments. Cells were washed and lysed in 10 mM Tris–HCl pH 7.4, 1% sodium dodecyl sulfate (SDS). Protein content in the cell lysate as well as in the supernatant was determined with the bicinchoninic acid method (BCA assay, Pierce, Rockford, IL). Twenty micrograms were mixed with sample buffer (0.03% bromophenol blue, 0.4 M Tris–HCl pH 7.4, 20% glycerol, 5% SDS) and separated on 10% SDS–polyacrylamide gels containing either gelatin (1 mg/ml) or β-casein (0.5 mg/ml). After electrophoresis, gels were washed for 1 h in renaturation buffer (2.5% Triton X-100 in aqua bidest.) and subsequently incubated for 36 h at 37°C in 50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.5. Gels were stained with 0.05% Coomassie Brillant Blue and destained with 30% methanol and 10% acetic acid, the clear zones within the blue background indicating proteinolytic activity. MMPs were identified by size and by comparison with the respective recombinant protein (Calbiochem-Novabiochem, Bad Soden, Germany) used as an additional marker. Quantification was performed by densitometry (software WinCam 2.2, Cybertech, Berlin, Gemany). All experiments were performed at least in triplicate.

MMP-2 and -9 activity

Biologically active MMP-2 and -9 antigen levels were measured in cell-culture supernatants using a MMP-2 and -9 (Amersham Bioscience, Freiburg, Germany) ELISA according to the manufacturer's instructions. The assay allows quantification of the active as well as the total protein. It has a sensitivity of <0.5 ng/ml. After the addition of chromogen, plates were read in a Dynatech MR 5000 plate reader at 405 nm.

Statistical analysis

Graphs were created with the sigma plot for windows graphic system (version 1.1, Jandel, Chicago, IL). Data were analysed with the Student's *t*-test. A *P*-value <0.05 was considered significant. Results are expressed as means with standard deviation (SD).

Results

In vitro invasiveness

To assess whether co-culture of tumour cells and macrophages increased invasion of tumour cells, we cultured the breast cancer cell lines MCF-7, SK-BR-3 and the benign mammary epithelial cell line hTERT-HME1 on EHS-matrix. Human macrophages, derived from peripheral blood monocytes, were placed in special inserts in the wells without direct cell-to-cell contact.

After 96 h of co-incubation with macrophages, the invasive capacity of MCF-7 and SK-BR-3 cells was significantly increased (Figures 1 and 2). Addition of either the

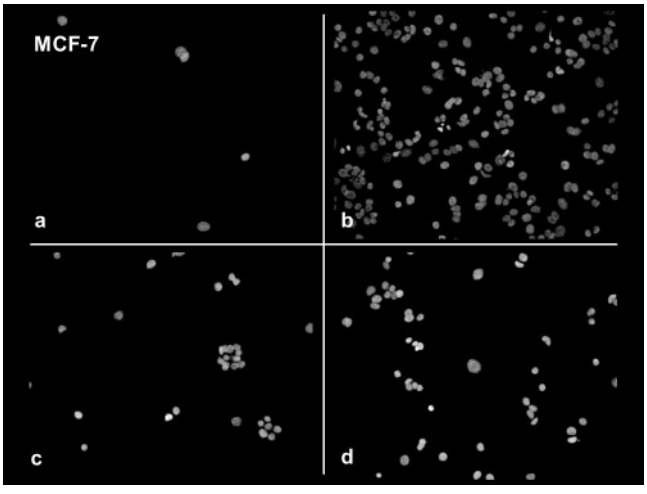

Fig. 1. Matrigel invasion assay. DAPI staining of migrated tumour cells. Representative ($n = 10$) sections are shown. (**a**) MCF-7 controls, (**b**) MCF-7 + macrophages, (**c**) MCF-7 + macrophages + neutralizing TNF-α-mAb, (**d**) MCF-7 + macrophages + MMP-9 mAb.

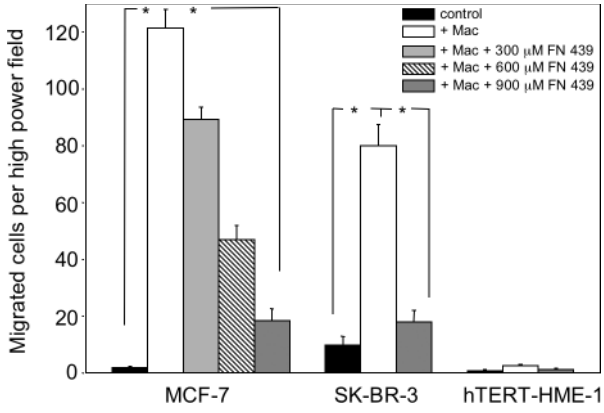

Fig. 2. Influence of co-cultivation with macrophages on invasiveness of MCF-7, SK-BR-3 and hTERT-HME1 cells in a Matrigel invasion assay. Results are shown as means ± SD ($n = 6$). Brackets and asterisks indicate statistically significant differences ($P < 0.0001$).

MMP inhibitor FN 439 or of the neutralizing TNF-α antibody to the co-culture reduced the enhanced invasiveness to almost the control levels (Figure 1c) ($P < 0.0001$). A slightly weaker reduction was achieved using a neutralizing antibody against MMP-9 (Figure 1d). The benign cell line hTERT-HME1 did not show any invasive behaviour either with or without macrophages (Figure 2).

Influence of LPS stimulation

To address whether activated and quiescent macrophages influence invasion differently, LPS was used as an inflammatory stimulus. Co-culture of LPS-activated macrophages enhanced invasiveness of the tumour cells [migrated MCF-7 cells per high power field: (21.3 ± 6.6; $n = 6$) to a lesser extent than quiescent macrophages (49.0 ± 4.9; $P < 0.0001$)]. The same results could be measured with SK-BR-3 cells while there was no effect on the hTERT-HME1 cell line (data not shown).

MMP protein expression and activity

In order to determine which cell types contributed to MMP secretion and subsequent invasion, we assessed MMP levels by zymography and carried out ELISA for active MMP-2.

The benign cell line hTERT-HME1 did not express any MMPs (data not shown). In control MCF-7 and SK-BR-3 cell extracts, only barely detectable levels of MMP-2/-9 and MMP-3/-7 could be measured, while slightly higher amounts of MMP-2 and -9 were secreted into the supernatant. Macrophages showed significant expression of all four above-mentioned MMPs in the cellular extracts as well as in the respective supernatant already under control conditions (Figures 3 and 4 and Tables I and II).

Upon co-incubation of tumour cells and macrophages, MMP-2 and -9 as well as MMP-3 and -7 concentrations in the joint supernatant were significantly increased (Figures 3 and 4 and Tables I and II). MMP-2 and -9 secretion could be down-regulated to control levels by addition of the neutralizing TNF-α antibody (Figure 5 and Table III). Co-culture of hTERT-HME1 and macrophages did not alter MMP concentrations either in the joint supernatant or in the cellular extracts (data not shown).

The increase of MMP-2 protein secretion upon co-culture of tumour cells with macrophages was paralleled by a significant increase in MMP-2 activity (Figure 6). While constitutive activity of MMP-2 in the supernatants of MCF-7, SK-BR-3

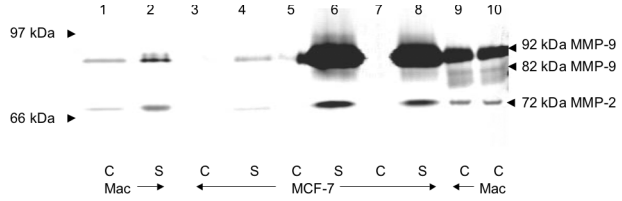

Fig. 3. Influence of co-cultivation of macrophages and MCF-7 cells on MMP-2 and -9 expression. Gelatin zymography of cells and supernatants from MCF-7 and macrophages, cultivated for 96 h either alone or together. C = cells, S = supernatant. Lane 1, macrophages, control (C); lane 2, macrophages, control (S); lane 3: MCF-7, control (C); lane 4, MCF-7, control (S); lane 5, MCF-7 (C) in co-culture with macrophages; lane 6, MCF-7 (S) in co-culture with macrophages; lane 7, MCF-7 (C) in co-culture with macrophages plus LPS; lane 8, MCF-7 (S) in co-culture with macrophages plus LPS; lane 9, macrophages (C) in co-culture with MCF-7; lane 10, macrophages (C) in co-culture with MCF-7 plus LPS (MW = molecular weight marker).

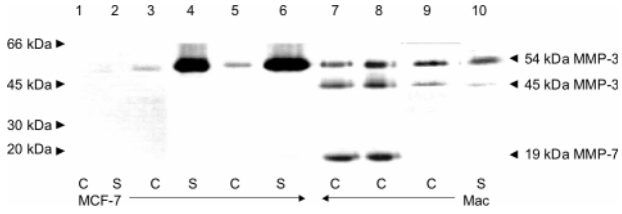

Fig. 4. Influence of co-cultivation of macrophages and MCF-7 cells on MMP-3 and -7 expression. Casein zymography of cells and supernatants from MCF-7 and macrophages, cultivated for 96 h either alone or together. C = cells, S = supernatant. Lane 1, MCF-7, control (C); lane 2, MCF-7, control (S); lane 3, MCF-7 (C) in co-culture with macrophages; lane 4, MCF-7 (S) in co-culture with macrophages; lane 5, MCF-7 (C) in co-culture with macrophages plus LPS; lane 6, MCF-7 (S) in co-culture with macrophages plus LPS; lane 7, macrophages (C) in co-culture with MCF-7; lane 8, macrophages (C) in co-culture with MCF-7 plus LPS; lane 9, macrophages, control (C); lane 10, macrophages, control (S) (MW = molecular weight marker).

Table I. Densitometric quantification of MMP-2 and -9 protein expression[a]

$n = 6$	Intensity (arbitrary units)		
	pro-MMP-9	MMP-9	pro-MMP-2
Mac, control (C)	6.45 ± 2.1	n.d.	6.23 ± 1.3
Mac, control (S)	8.03 ± 1.5	n.d.	7.30 ± 1.4
MCF-7, control (C)	n.d.	n.d.	n.d.
MCF-7, control (S)	4.92 ± 1.2	n.d.	4.16 ± 1.2
MCF-7 + Mac (C)	n.d.	n.d.	n.d.
MCF-7 + Mac (S)	83.19 ± 3.6	4.23 ± 0.8	41.57 ± 2.6
MCF-7 + Mac + LPS (C)	n.d.	n.d.	n.d.
MCF-7 + Mac + LPS (S)	84.04 ± 2.3	3.99 ± 1.3	31.21 ± 2.8
Mac, co-culture (C)	55.90 ± 3.5	1.02 ± 0.3	3.86 ± 1.4
Mac + LPS, co-culture (C)	51.55 ± 2.4	0.86 ± 0.2	3.91 ± 1.6

[a]Means ± SD.
C = cells; S = supernatant; n.d. = not detectable; Mac, macrophages.

and macrophages was low, the enzyme activity was significantly up-regulated under co-culture conditions. As expected, addition of the broad-spectrum antagonist FN 439 inhibited MMP-2 activity. Similar results were obtained with MCF-7 and macrophages, while hTERT-HME1 did not react to co-cultivation with enhanced enzyme activity. Analogous results could be shown for MMP-9 activity (macrophages,

Table II. Densitometric quantification of MMP-3 and -7 protein expression[a]

$n = 6$	Intensity (arbitrary units)			
	pro-MMP-3	MMP-3	pro-MMP-7	MMP-7
MCF-7, control (C)	n.d.	n.d.	n.d.	n.d.
MCF-7, control (S)	n.d.	n.d.	n.d.	n.d.
MCF-7 + Mac (C)	2.51 ± 1.1	n.d.	n.d.	n.d.
MCF-7 + Mac (S)	37.27 ± 2.5	n.d.	n.d.	1.78 ± 1.5
MCF-7 + Mac + LPS (C)	6.74 ± 1.6	3.25 ± 2.1	3.01 ± 1.1	2.13 ± 1.9
MCF-7 + Mac + LPS (S)	38.38 ± 3.5	n.d.	n.d.	n.d.
Mac, co-culture (C)	5.83 ± 2.1	n.d.	n.d.	n.d.
Mac, co-culture + LPS (C)	37.10 ± 2.3	n.d.	n.d.	1.44 ± 2.1
Mac, control (C)	12.61 ± 2.3	12.75 ± 2.6	n.d.	8.45 ± 1.4
Mac, control (S)	13.99 ± 2.7	14.21 ± 3.8	n.d.	10.22 ± 2.6

[a]Means ± SD.
C = cells; S = supernatant; n.d. = not detectable; Mac, macrophages.

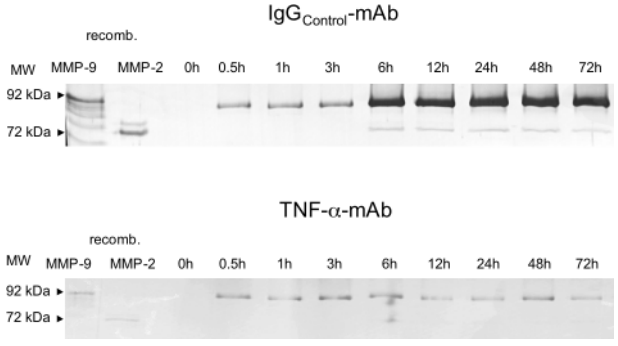

Fig. 5. Influence of TNF-α-neutralization on MMP-2 and -9 expression of macrophages in co-culture with MCF-7 cells. First lane, gelatin zymography of the joint supernatant with addition of an IgG-control antibody; second lane, gelatin zymography of the joint supernatant with addition of a neutralizing TNF-α-antibody.

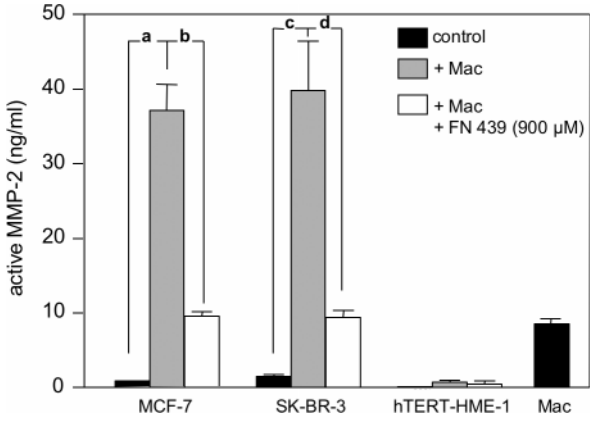

Fig. 6. MMP-2 activity in the supernatant of MCF-7, SK-BR-3 and hTERT-HME1 cells, cultivated either alone or together with macrophages ± the MMP-inhibitor FN 439 (ELISA). Results are shown as means ± SD ($n = 8$). The following differences were statistically significant: a, $P = 0.0002$; b, $P = 0.0005$; c, $P = 0.0013$; d, $P = 0.0033$.

Table III. Quantification of MMP-2 and -9 down-regulation by TNF-α neutralization (MMP-2 and -9 ELISA of the joint supernatant, total protein)[a]

Concentration of neutralizing TNF-α-mAb (pg/ml)	Total protein (ng/ml)	
	MMP-2	MMP-9
0	7.65 ± 1.62	11.70 ± 1.68
1	6.62 ± 0.66	10.83 ± 1.31
10	6.39 ± 0.56	10.86 ± 0.45
100	4.34 ± 0.31	7.92 ± 1.38
250	2.98 ± 0.30	6.01 ± 0.28
500	2.14 ± 0.17	4.91 ± 1.02
1000	1.94 ± 0.52	2.83 ± 0.38

[a]Means ± SD (ng/ml).

3.49 ± 0.31 ng/ml; MCF-7, 0.16 ± 0.02 ng/ml; macrophages + MCF-7, 11.01 ± 2.10 ng/ml).

MMP-mRNA expression

To further define the source of the increased MMP production, MMP-mRNA was quantified in the various cellular components of the co-culture system. Under control conditions, macrophages expressed mRNAs of MMP-1, -2, -3, -7, -9, -12 and -14, some of them only weakly. Upon co-culture with tumour cells, expression of MMP-2 and -9 increased significantly

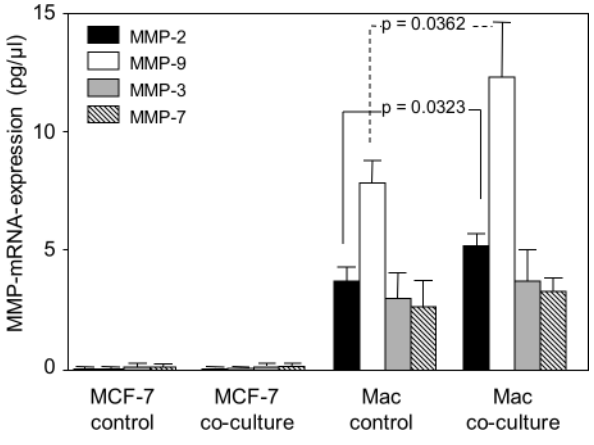

Fig. 7. MMP mRNA expression in MCF-7 cells and macrophages, cultured either alone or together. Quantitative real-time RT–PCR on the Light Cycler System® (means ± SD, $n = 8$).

(Figure 7), while MMP-3 and -7 mRNA concentrations, in contrast to the zymography results, remained constant. Addition of the TNF-α antibody reduced MMP-2 and -9-expression in co-cultured macrophages in a dose-dependent

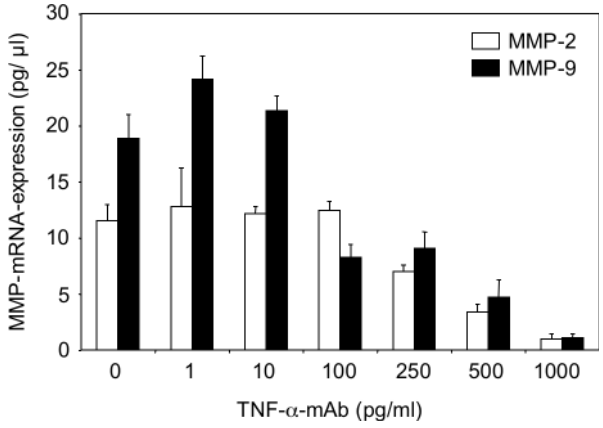

Fig. 8. Influence of various concentrations of a neutralizing TNF-α antibody on the mRNA-expression of MMP-2 and -9 in macrophages, which had been co-cultivated with MCF-7 cells (quantitative real-time RT–PCR).

manner (Figure 8). Co-culture of macrophages with hTERT-HME1 did not influence MMP-expression pattern of the macrophages. The respective mRNA concentrations in the tumour cell lines and the benign mammary epithelial cell line were below a cut-off of 100 fg and remained unchanged upon exposure to macrophages (data not shown).

TNF-α expression and regulation

As assessed by multiplex real-time PCR, TNF-α mRNA was mainly expressed by the macrophages while the mRNA concentration in the tumour cells was low (compared with the macrophages). Upon co-culture with tumour cells the TNF-α mRNA concentration increased significantly in the macrophages, the respective mRNA in the tumour cells remained constant (Figure 9A). Co-cultivation of tumour cells with macrophages increased the amount of TNF-α in the joint supernatant. This could be reduced by adding the MMP inhibitor FN 439 in high concentrations (900 µM) (Figure 9B).

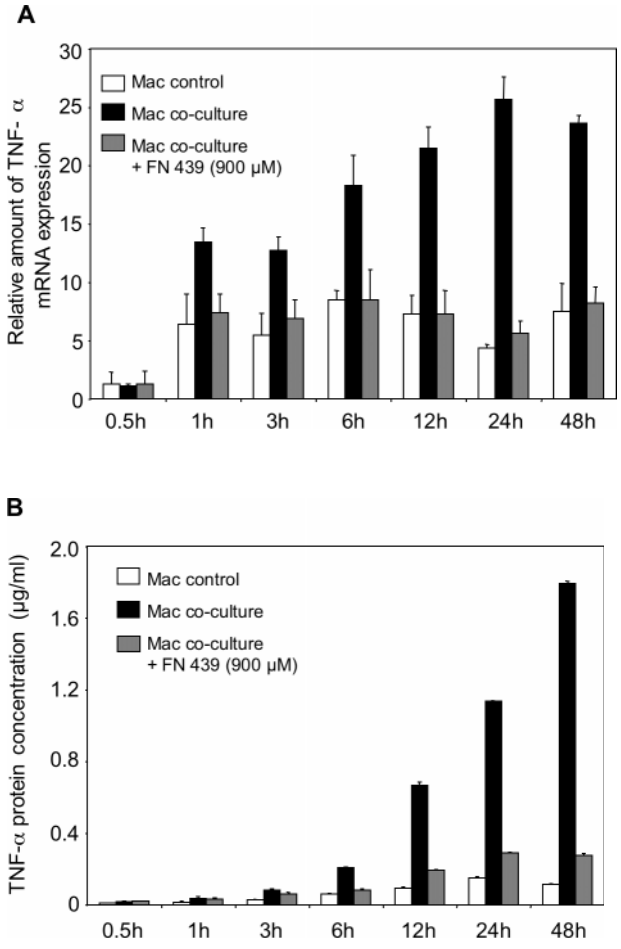

Fig. 9. (**A**) TNF-α mRNA expression in macrophages as well as in macrophages co-cultivated with MCF-7 cells ± the MMP-inhibitor FN 439. The bars show the relative amount of TNF-α mRNA normalized to the amount of 18S rRNA. (**B**) TNF-α protein concentration in the supernatant of macrophages as well as of macrophages co-cultivated with MCF-7 cells ± the MMP-inhibitor FN 439 (ELISA).

Discussion

The presented data support the hypothesis that not only the malignant cells themselves, but also the macrophages as a component of the benign stromal compartment, are involved in the regulation of tumour cell invasion.

Co-culture of macrophages with two breast cancer cell lines lead to a significant increase in the *in vitro* invasiveness of the tumour cells. The enhanced invasion rate was correlated with an induction of MMPs, especially of MMP-2, -3, -7 and -9. Invasion was diminished by addition of a neutralizing MMP-9 antibody and almost completely abrogated by the broad-spectrum MMP antagonist FN 439. Analysis of mRNA expression in the various cells revealed the macrophages as the main source of MMP production. In contrast to MMP-2 and -9, up-regulation of MMP-3 and -7 protein was not accompanied by induction of the respective mRNA. This obvious discrepancy may point to additional post-transcriptional and translational regulatory mechanisms, but may also be explained by a very early up-regulation during the first hours of exposure to macrophages, which may have escaped detection.

It is not surprising, that MMP up-regulation leads to enhanced invasiveness of breast cancer cells. There is considerable evidence in the literature that MMPs are essential for tumour growth, invasion and metastasis (30,31). For example, targeted over-expression of MMP-3 in transgenic mice gave rise to pre-neoplastic and malignant mammary gland tumours (32,33). Clinically, high levels of MMP-2 and -3 have been found to correlate with poor outcome in patients with breast cancer (21,22,31).

MMPs do not necessarily originate from the malignant compartment of the tumour, but also from benign cells such as fibroblasts (21,34). Especially fibroblasts growing in the neighbourhood of tumour cells, so-called tumour-associated fibroblasts are able to stimulate tumour cell motility and invasion (35). Direct co-cultivation of ovarian cancer cells with fibroblasts induced MMP-2 release in the latter and led to an enhanced invasion of the former (36,37).

There are only few data about the role of the macrophages in this context. Although macrophages, in principle, are tumouricidal and part of the host's antitumour defence mechanisms (4), there is considerable evidence that macrophages may not only fail to kill tumour cells but even contribute to tumour progression. Focal macrophage infiltration appears to be an important prognostic factor in invasive carcinoma of the breast, high infiltration rates predicting reduced relapse-free and overall survival (6). In transgenic mice susceptible to breast cancer development, which were crossed with mice

deficient of macrophage colony-stimulating factor 1 (CSF-1), tumour cell invasion was significantly reduced, whereas overexpression of CSF-1 in these mutants accelerated tumour cell dissemination and was associated with high macrophage infiltration (38).

In accordance with our results, co-culture of the ovarian cancer cell line PEO-1 and the monocytic cell line THP-1 led to an up-regulation of monocyte MMP-9 production (39). Swallow and co-workers demonstrated enhanced MMP-2 release by human monocytes upon direct cell-to-cell contact with metastatic colorectal cells (24).

In the presented microinvasion model, MMP induction was dependent on up-regulation of TNF-α, which was produced by the macrophages in response to co-cultivation with tumour cells. Addition of a neutralizing TNF-α antibody down-regulated increased invasiveness of the co-cultivated tumour cells as well as the expression of MMP-2 and -9 mRNA in the macrophages. TNF-α release was reduced as a consequence of MMP inhibition. This may at least partially be explained by decreased TNF-α shedding at the cellular surface, suggesting that TNF-α represents a part of an autoregulatory loop.

The finding that TNF-α plays a major role in this context is supported by data from other authors. TNF-α has been found to be involved in chemokine stimulation of MMP-9 in monocytes (25) as well as in ovarian carcinoma cell-induced up-regulation of monocyte MMP-9 production (39). A potential tumour-promoting effect of TNF-α has been demonstrated by Moore and co-workers, showing that mice deficient in TNF-α were resistant to skin carcinogenesis (40). Overexpression of TNF-α increased metastatic activity of tumour lines (41), and treatment of mice with TNF-α promoted development of liver metastases (42).

It is still unknown how tumour cells interact with macrophages to stimulate them to produce TNF-α as well as MMPs and to facilitate tumour cell invasion. As there was no direct cell-to-cell contact in the presented co-culture model, one or several soluble molecules are to be postulated as stimulating factors. Probable candidates are cytokines like IL-4, -6 and -10 (43–45) and CC-chemokines, such as CCL2 (MCP-1), 3 (MIP-1a) or 5 (RANTES) (25), all of which have been shown to be involved in monocyte MMP release. However, it seems unlikely, that a typical inflammatory profile of cytokines leads to TNF-α up-regulation and MMP induction, as we could demonstrate that LPS-activated macrophages had a weaker effect on invasiveness than quiescent ones.

In conclusion, the *in vitro* interaction of breast cancer cells with macrophages via still to be identified soluble compounds induces macrophages to release MMPs in a TNF-α dependent way and to support tumour cell invasion. Further experiments are needed to better define how these interactions are regulated and to demonstrate whether similar effects play a role *in vivo*.

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