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UPREGULATION OF MATRIX METALLOPROTEINASE-9 IN MURINE 5T33 MULTIPLE MYELOMA CELLS BY INTERACTION WITH BONE MARROW ENDOTHELIAL CELLS

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Abstract: MM is a B-cell malignancy mainly characterized by monoclonal expansion of plasma cells in the BM, presence of paraprotein in serum and occurrence of osteolytic bone lesions. MMPs are a family of proteolytic enzymes that can contribute to cancer growth, invasion, angiogenesis, bone degradation and other processes important in the pathogenesis of MM. We investigated MMP-9 production in the 5T33MM murine model. Expression of MMP-9 protein in supernatant and cell extracts was analyzed by gelatin zymography. The *in vitro,* stroma-independent variant 5T33MMvt showed no protein expression of MMP-9 in contrast to *in vivo* growing MM cells, 5T33MMW. However, when 5T33MMvt cells were injected into naive mice and isolated after tumor take (5T33MMvt-vv), they secreted a significant amount of MMP-9. These results were confirmed by specific staining of cytospins with an anti-MMP-9 antibody. The MMP-9 production by 5T33MMvt-vv cells disappeared when the cells were recultured *in vitro.* These data demonstrated that upregulation of MMP-9 occurs *in vivo* and that this process is dependent on the microenvironment. Cocultures of 5T33MMvt cells with STR10 BMECs induced MMP-9 in MM cells, as determined by both gelatin zymography and flow-cytometric analysis. In conclusion, our results demonstrate that MMP-9 production by MM cells is upregulated *in vivo* by the interaction of MM cells with BMECs.

Key words: multiple myeloma; matrix metalloproteinase-9; bone marrow; stromal cell; endothelial cell

Abbreviations: BM, bone marrow; BMEC, bone marrow endothelial cell; BMSC, bone marrow stromal cell; C_v cycle threshold; ECM, extracellular matrix; FAM,6-carboxyfluorescein; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LN, laminin; MCP-1, monocyte chemoattractant protein-1; MM, multiple myeloma; MMP, matrix metalloproteinase; PBMC, peripheral blood mononuclear cell; SDF, stromal cell-derived factor; TBS, TRIS-buffered saline; TIMP, tissue inhibitor of matrix metalloproteinase.

MMPs are a family of zinc-dependent endopeptidases involved in the degradation of many components of the ECM and the basement membrane.[1,2] Depending on their substrate specificity and structure, members of the MMP family can be divided into subgroups of collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10, -11, -7), gelatinases (MMP-2, -9), membrane-type MMPs and other MMPs.[2,3] MMPs are secreted as inactive proenzymes and activated extracellularly by proteolytic cleavage. MMP production is regulated at the level of transcription, secretion and/or activation.[3,4] All MMPs are inhibited by specific TIMPs, including TIMP-1, -2, -3 and -4. TIMP-1 and TIMP-2 bind tigthly with, respectively, pro-MMP-9 and pro-MMP-2 to regulate their activity.[5] The balance between MMP and TIMP levels determines the net proteolytic activity. This equilibrium is highly regulated in physiologic conditions, *e.g.,* wound healing and embryogenesis,[3] but is disturbed in pathologic circumstances, *e.g.,* rheumatoid arthritis, multiple sclerosis and cancer.[6-9]

MM is a B-cell cancer mainly characterized by proliferation of malignant plasma cells in the BM, presence of a monoclonal serum immunoglobulin and occurence of osteolytic lesions. The disease occurs at older ages and remains incurable despite progress in treatment. Therefore, new approaches for therapy are necessary. The hypothesis is that MMPs are involved in a number of events underlying MM progression, *e.g.,* transendothelial migration, invasion, osteolytic lesions and angiogenesis.[10] Barillé *et al.*[11] demonstrated MMP-9 production by human MM cells. MMP-9 is involved in transendothelial migration and invasion.[12,13] This suggests a role of the protease in the homing of MM cells to the BM, which implicates transendothelial migration and invasion of MM cells in the BM. Coculture of malignant plasma cells and BMSCs induces MMP-1 and activates MMP-2. MMP-7, secreted by MM cells, is responsible for MMP-2 activation.[14] Since MMP-1 has a major structural component of the bone, collagen I, as substrate, it can be involved in the initiation of osteolytic lesions. Formation of new

blood vessels, called *angiogenesis,* is induced in MM both in human MM samples[15] and in the 5TMM murine model.[16] Studies with MMP inhibitors have demonstrated the role of MMPs in angiogenesis.[17-20] MMPs may also be indirectly involved in MM progression by the release of cytokines, growth factors and angiogenic factors stored in the ECM of the BM.

To elucidate the regulation of MMPs in MM, we used the murine 5T33MM model. The 5TMM cell lines initially originated spontaneously in aging C57Bl/KaLwRij mice and have since been propagated in young syngeneic mice by i.v. transfer of the diseased BM. By this method, several 5TMM models were developed with characteristics similar to the human disease.[21] Mice develop MM spontaneously at older ages, MM cells are localized in the BM, tumor load can be assessed by paraproteinemia, osteolytic lesions develop and the molecular mechanisms are similar to those in humans.[22-25] The 5T33MM mouse model represents a rapidly progressive variant of MM.[22,24] A BM stroma-independent variant of 5T33MMVV cells, the 5T33MMvt cell line, was obtained. *In vivo* and *in vitro* 5T33MM cells have identical idiotypic immunoglobulin sequences, demonstrating their common clonal origin.[26] These 2 cell lines are good tools to study several molecules important in MM pathogenesis.

We investigated the production and regulation of MMP-9 in the 5T33MM model. MMP-9 was secreted by *in vivo* growing 5T33MM cells but not by the *in vitro* stroma-independent variant of 5T33MM. However, when 5T33MMvt cells were injected into naive mice, the MM cells isolated from the BM (5T33MMvt-vv) after tumor take showed upregulated MMP-9 secretion. Once these cells were cultured again *in vitro,* MMP-9 production decreased and ultimately disappeared, indicating the BM microenvironmental dependence of MMP-9 production. Interestingly, the *in vitro* interaction of 5T33MM cells with BMECs resulted in upregulation of MMP-9 in 5T33MM cells.

MATERIAL AND METHODS

Mice

C57BL/KaLwRij mice were purchased from Harlan (Horst, the Netherlands). They were housed and treated following the conditions approved by the Ethical Committee for Animal Experiments of the Free University of Brussels (license LA1230281).

Cell lines

Various 5T33MM cell lines were used. For the nomenclature of the different cell lines, we used *vv, vt* and *vt-vv,* which stand, respectively, for *vivo, vitro* and *vitro-vivo.* The 5T33MMvv cell line originated from elderly C57B1/KaLwRij mice that spontaneously developed MM. The cells have since been propagated in young syngeneic mice by i.v. transfer of the diseased BM.[21] Disease progression was followed up by protein electrophoresis of serum samples.[24] Mice were killed when a serum paraprotein concentration of 10 mg/ml was reached. The BM was isolated and the MM cells were purified as previously described.[16] Cell purity was determined by FACS analysis.[24]

The 5T33MMvt cell line, obtained by the Radl group, resulted spontaneously from cultured 5T33MMvv cells and grows *in vitro* independent of the BM stroma. Cells were cultured in RPMI-1640 medium (GIBCO-Life Technologies, Ghent, Belgium) supplemented with penicillin-streptomycin, glutamine, MEM (all from GIBCO-Life Technologies) and 10% serum (Fetal clone I; Hy-clone, Logan, UT) (complete growth medium). 5T33MMvt cells inoculated *in vivo* and isolated after tumor take are called *5T33MMvt-vv* cells.

Mouse BMECs, STR10, and lung endothelial cells, LE1SVO (kindly provided by Dr. M. Kobayashi, Japan), were cultured in RPMI-1640 medium supplemented as described above.

Subcloning of 5T33MMvt-vv cells

Freshly isolated BM cells of a 5T33MM-bearing mouse were seeded in round-bottomed, 96-well plates at 0.3 cells/well in 100 µl medium. Culture medium was RPMI supplemented with penicillin-streptomycin, glutamine, MEM and 5% FCS. After 3 days, 10% hybridoma cloning factor (Origen; Sanvertech, Boechout, Belgium) was added. Once a week, 50 µl culture supernatant were replaced by 50 µl fresh medium plus hybridoma cloning factor. After 10 days, clones were visible in 14/288 wells. These clones were expanded in 24 wells.

Gelatin zymography

Cells were plated at a density of 2×10^5/well on a 24-well plate in 300 µl serum-free RPMI medium. Supernatant was harvested after 18 hr and electrophoresed under nonreducing conditions using 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Merck, Darmstadt, Germany). After electrophoresis, the gel was washed in 2% Triton X-100 for 30 min twice to remove SDS. After overnight incubation in 0.05 M Tris buffer (pH 7.6) containing 10 mM $CaCl_2$ and 3 mM NaN_3, gels were stained with Coomassie brilliant blue and destained in 40% methanol/10% acetic acid. Gelatinolytic activity was identified as a clear band on a blue background. Supernatant from human fibrosarcoma HT1080 cells was used as positive control for the detection of MMP-2 and MMP-9. The 72 kDa band corresponds to MMP-2, the 92 kDa band to MMP-9. Mouse MMP-9 has m.w. 110 kDa and is thus located higher in the gel than human MMP-9 (92 kDa). The density of the MMP-9 band was measured with the NIH (Bethesda, MD) 1.62 image program.

MMP-9 immunostaining on cytospins

Cytospins of 5T33MM cell suspensions (4×10^5 cells/ml) were prepared at 72 g during a 7 min spin (Cytospin-2; Shandon, London, UK). Cytospins were fixed for 5 min in 1% paraformaldehyde in 0.07 M sodium phosphate buffer (pH 7.0) and rinsed 3 times for 5 min in TBS (0.01 M TRIS buffer containing 0.9% NaCl and 0.1% Triton X-100, pH 7.6). This was followed by preincubation with preimmune swine serum (1/5 diluted in TBS containing 2% BSA) for 30 min. The first antibody, rabbit antimouse MMP-9 (17.5 µg/ml; kindly provided by Dr. P. Carmeliet, Leuven, Belgium), was incubated overnight at room temperature. Cytospins were rinsed in TBS and incubated with 0.3% H_2O_2 for 25 min. After rinsing again in TBS, peroxidase-labeled swine anti-rabbit IgG antibodies, at a dilution of 1:50, were incubated for 1 hr, followed by rinsing with TBS and PBS. Chromogenic visualization was accomplished using AEC+ (Dako, Glostrup, Denmark). Cells were then briefly counterstained in Harris hematoxylin and mounted with Aqua Poly/Mount (Polysciences, Warrington, PA).

Cocultures of endothelial cells with 5T33MMvt cells

STR10 and LE1SVO cells (2×10^5/well) were seeded onto 24-well plates in complete growth medium. After 24 hr, cells were washed twice with serum-free medium and cultured alone or with 5T33MMvt cells (2×10^5/well) in 300 µl serum-free medium. In some experiments, an insert (Transwell; Costar, Cambridge, MA) with 0.4 µm pore size was placed in wells with STR10 cultures to avoid direct contact between EC and MM cells. 5T33MMvt cells (2×10^5) in 100 µl serum-free medium were added to the upper compartment of the insert, and 200 µl serum-free medium were added to the well. After 48 hr incubation, culture supernatant was harvested and used for zymography.

Effect of growth factors and ECM proteins on MMP-9 expression

5T33MMvt cells were plated in 96-well plates at a density of 4×10^5/well and treated with SDF-1α (20-100 ng/ml) and SDF-1β (20-100 ng/ml) alone or together, IGF-I (0.1-1,000 ng/ ml), IL-6 (1-100 ng/ml), MCP-1 (1-100 ng/ml), LN (0.1-10 µg/ml) and FN (0.005-5 µg/ml) in serum-free RPMI medium for 48 hr, followed by gelatin zymography. SDF-1α, SDF-1β, MCP-1 and IL-6 were purchased from Preprotech (Rocky Hill, NI). LN and FN were, respectively, purchased from Sigma-Aldrich (Bornem, Belgium) and Invitrogen (Groningen, the Netherlands).

Flow cytometry

To assess MMP-9 expression in STR10 and 5T33MMvt cells, cells were detached with cell dissociation solution (Sigma-Aldrich) and intracytoplasmatically stained with MMP-9 antibodies. Cells were first fixed for 10 min in 4.5% formaldehyde and 22% (v/v) acetone in FACS flow (Becton Dickinson, Mountain View, CA) and washed with FACS flow. This was followed by perme-abilization of cells with 1% saponin and 10% BSA in FACS flow and incubation with rabbit antimouse MMP-9 antibodies for 15 min. After washing, cells were again fixed, permeabilized and incubated with the second antibody, donkey antirabbit IgG conjugated to FITC (Jackson Immunoresearch, West Grove, PA).

For cocultures (STR10 + 5T33MMvt), intracytoplasmatic double staining for MMP-9 and anti-5T33MM idiotype was performed with MMP-9 antibodies and anti-idiotypic antibodies.[24] Rat antimouse IgG1-phycoerythrin (Becton Dickinson) was used as a second step for 5T33MM idiotype detection.

To assess syndecan-1 (CD138) expression in STR10 and 5T33MMvt cells before and after coculture, cells were detached with cell dissociation solution and membranes stained with CD138 antibodies (Pharmingen, San Diego, CA). For all stainings, iso-type-matched irrelevant antibodies were used as controls.

RNA isolation and cDNA synthesis

Total RNA from 5×10^6 5T33MM cells was isolated using the SV total RNA isolation system (Promega, Madison WI) according to the manufacturer's instructions. The concentration and purity of RNA were determined by spectrophotometric measurement (Gene Quant II; Pharmacia Biotech, Cambridge, UK). Total RNA (1-5 μg) was converted into cDNA by the superscript first-strand synthesis system (GIBCO-Life Technologies) using random hexamers as primers.

Real-time quantitative PCR

The principle of real-time quantitative PCR is the use of a dual-labeled fluorogenic probe that contains a fluorescent reporter dye at the 5 ' end and a quencher dye at the 3 ' end. The probe binds specificially to its PCR fragment. When the probe is intact, emission from the reporter is quenched by the quencher. During the extension phase of PCR, the annealed probe is cleaved by the 5 ' nuclease activity of Taq polymerase, thereby releasing the reporter from the quencher. This results in an increase of fluorescence emission of reporter dye, which was detected quantitatively by the ABI (Foster City, CA) PRISM 7700 Sequence Detector. The amount of fluorescence measured in a sample was proportional to the amount of specific PCR product generated. By analysis of data, the C_t value was determined. This parameter represents the PCR cycle at which an increase in fluorescence is detected above a baseline signal.

Primers and probes for the detection of MMP-9 mRNA were designed using the computer program Primer Express (ABI). Forward and reverse primers used for the amplification of MMP-9 were 5'-ACCCGAAGCGGACATTGTC-3' and 5'-CGAAGG-GATACCCGTCTCC-3', respectively. Amplicon length was 62 bp. The sequence of the MMP-9 Taqman probe was 5'-TC-CAGTTTGGTGTCGCGG-3'. Primers were located in 2 different exons to eliminate detection of genomic DNA. To standardize the amount of sample RNA, we amplified an endogenous reference gene, *GAPDH.* Primers and probes for *GAPDH* were purchased from ABI and used as described by the manufacturer. The GAPDH and MMP-9 probes were labeled with a quencher dye (TAMRA) and a reporter dye (FAM for MMP-9, VIC for GAPDH). Taqman PCR was performed in a 25 μl reaction mix containing 2.5 μl 10 × real-time PCR buffer, 5 mM $MgCl_2$, 200 μM dNTPs, 50 nM forward primer, 900 nM reverse primer, 200 nM Taqman probe, 0.25 U AmpErase uracil-*N*-glycosylase, 1.25 U HotGoldStar (ABI) and 2.6 μl cDNA for the detection of MMP-9 mRNA. Samples were amplified by 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Each sample was amplified in triplicate.

The relative standard curve method was used to quantitate the relative MMP-9 mRNA expression in 5T33MM cells. Relative standard curves (50, 5, 0.5, 0.05 ng) were prepared for MMP-9 using cDNA from normal BM diluted with control rodent RNA (ABI) and for GAPDH using cDNA made from control rodent RNA.

Data analysis

The relative standard curve method is extensively described elsewhere.[27] Standard curves and line equations were generated in Excel (Microsoft Corporation, Redmond, WA) by plotting C_t values *(y* values) with the log input amount of RNA *(x* values). The input amount of MMP-9 and GAPDH mRNA of unknown samples (5T33MMvt, 5T33MMVV, 5T33MMvt-vv and normal BM) was calculated with the following formula: *log input amount* = (C_t value - *b)/m,* where *b* is the *y* intercept of the standard curve line, *m* is the slope of the standard curve line and *input amount* = $10^{\text{log input amount}}$

For each sampe, the input amount of GAPDH and MMP-9 mRNA was calculated in triplicate and then averaged. Next, the amount of MMP-9 mRNA was divided by the amount of GAPDH mRNA to determine the normalized amount of MMP-9 mRNA.

MMP-9 expression in *in vivo* samples was partially due to MMP-9 expression of normal cells. Therefore, MMP-9 expression from normal BM was subtracted from normalized amounts of the 5T33MMVV and 5T33MMvt-vv samples. The 5T33MMvt sample was chosen as the calibrator, or 1 × sample. To generate the relative expression of MMP-9 mRNA, normalized MMP-9 amounts of 5T33MMvv and 5T33MMvt-vv samples were divided by the 5T33MMvt normalized MMP-9 value.

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Statistical analysis

For statistical analysis, the Wilcoxon signed rank test was used. $p < 0.05$ was considered significant.

RESULTS

Expression of MMP-9 in 5T33MM cells

Basal expression of MMP-9 in culture supernatant and cell lysates of *in vivo* growing MM cells (5T33MMvv) and the *in vitro* stroma-independent variant (5T33MMvt) was examined by both gelatin zymography and immunostaining with anti-MMP-9 antibodies. 5T33MMVV cells produced MMP-9 in cell lysates (Fig. 1b) and secreted MMP-9 in the medium (Fig. 1a), in contrast to 5T33MMvt cells. No MMP-2 band was detected, indicating that 5T33MMvt and 5T33MMvv cells do not secrete MMP-2. To confirm the results obtained with zymography, cytospins of 5T33MMvt and 5T33MMvv cells were prepared and stained with a specific anti-MMP-9 antibody. Positive staining of 5T33MMvv cells (Fig. 2b), but not of 5T33MMvt cells (Fig. 2a), for MMP-9 is shown.

FIGURE 1: *Gelatin zymography of MMP-9 production by 5T33MM cells. Conditioned medium of HT1080 cells was used as positive control (lane 1) and serum-free RPMI medium as negative control (lane 2). Lane 3 represents 5T33MMvt and lane 4 5T33MMvv. (a) Gelatin zymography of 18 hr serum-free conditioned media, (b) Gelatin zymography of cell lysates. Results from 1 representative experiment of 3 are shown.*

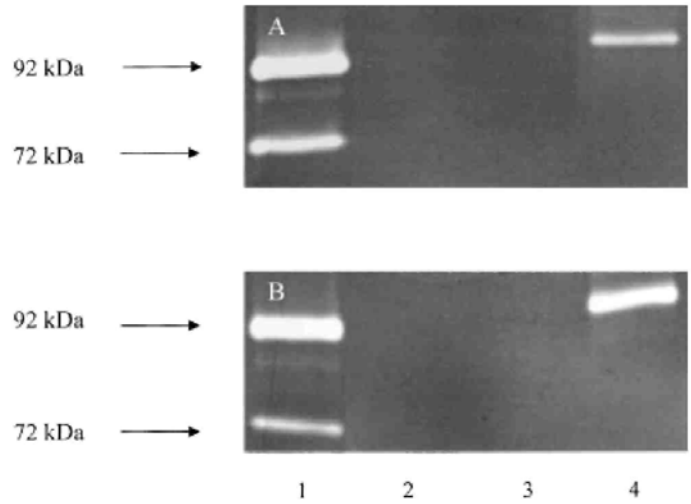
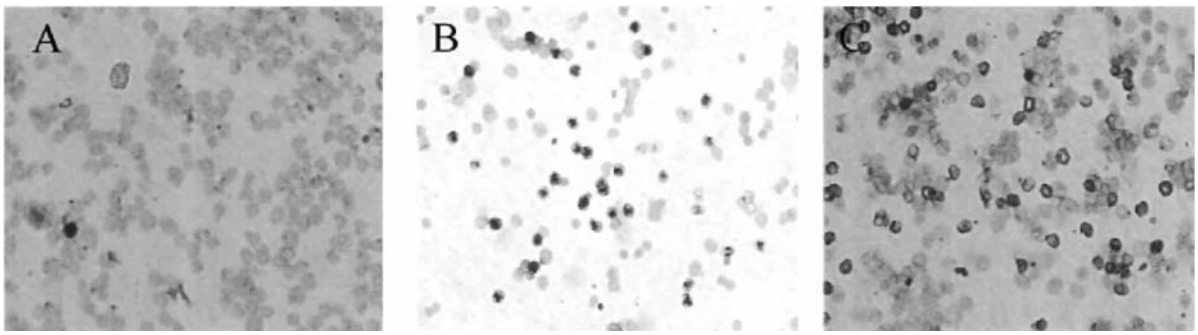


FIGURE 2: *MMP-9 immunostaining of 5T33MMvt (a), 5T33MMvv (b) and 5T33MMvt-vv (c) cells on cytospins. Original magnification x200.*



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MMP-9 upregulation is dependent on BM microenvironment

To demonstrate the *in vivo* upregulation of MMP-9, we injected 5T33MMvt cells i.v. into naive mice. After tumor take, 5T33MMvt-vv cells were harvested from the BM and analyzed for MMP-9 expression. Both gelatin zymography (Fig. 3*a*, lane 3) and immunostaining (Fig. 2*c*) demonstrated that 5T33MMvt-vv cells were positive for MMP-9. To investigate whether the MMP-9 upregulation was constitutive or dependent on the BM microenvironment, 5T33MMvt-vv cells were maintained in culture for 10 weeks (5T33MMvt-vv-vt). Secretion of MMP-9 was downregulated within 2 weeks and disappeared completely after 4-10 weeks (Fig. 3*a*). These results suggest that the BM microenvironment is important for MMP-9 induction *in vivo.* Next, 5T33MMvt-vv-vt cells were subcloned to investigate whether the downregulation of MMP-9 is due to selection of a subpopulation lacking MMP-9 secretion. Several clones were tested for MMP-9 production by zymography. None of the clones secreted MMP-9 (Fig. 3*b*).

In vivo cells were isolated from the BM and purified as previously described.[16] The purity of the *in vivo* cells was 80-97%. To check the contribution of MMP-9 production by 3-20% of contaminating cells, BM from a normal mouse was isolated. Conditioned medium from 2×10^5, 1×10^5, 0.5×10^5, 0.4×10^5, 0.3×10^5, 0.2×10^5 and 0.1×10^5 cells was tested by gelatin zymography (Fig. 4). No MMP-9 was detected in media conditioned by 0.4×10^5 cells (corresponding to 20% contamination in purified 5T33MM *in vivo* samples). These results demonstrate that MMP-9 production in *in vivo* MM cells is mainly due to MMP-9 secretion by the tumor cells themselves.

FIGURE 3: *Gelatin zymography of MMP-9 upregulation in vivo and BM microenvironment dependence. Lanes 1 and 2 are positive (conditioned medium of HT1080 cells) and negative (serum-free medium) controls, respectively, (a) Gelatin zymography of supernatant of 5T33MMvt-vv ceUs (lane 3) and of 5T33MMvt-vv cells cultured in vitro for 2 weeks (lane 4) and 4 weeks (lane 5). Results from 1 representative experiment of 3 are shown, (b) Gelatin zymography of supernatant of different 5T33MMvt-vv-vt clones (lanes 3-8).*

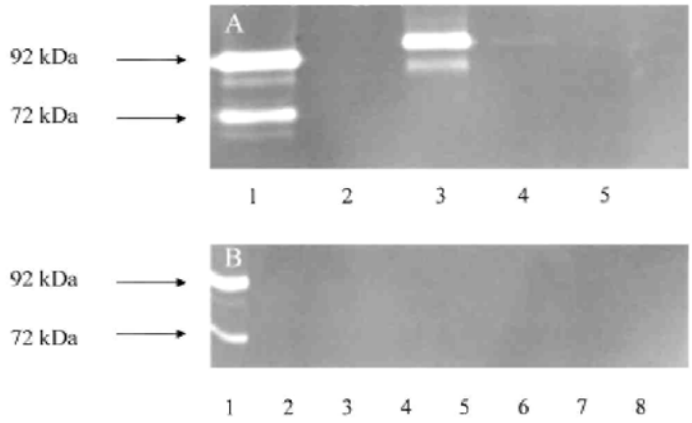
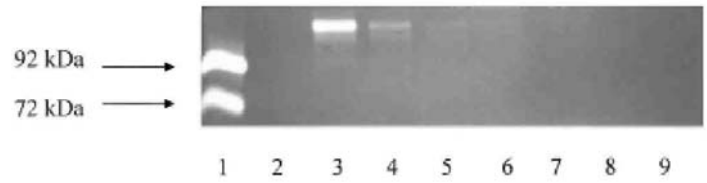


FIGURE 4: *Gelatin zymography showing MMP-9 secretion by 2×10^5 (lane 3), 1×10^5 (lane 4), 0.5×10^5 (lane 5), 0.4×10^5 (lane 6). 0.3×10^5 (lane 7), 0.2×10^5 (lane 8) and 0.1×10^5 (lane 9) cells of normal BM representing, respectively, 100%, 50%, 25%, 20%, 15%, 10% and 5% of contamination in the conditioned medium of in vivo samples.*



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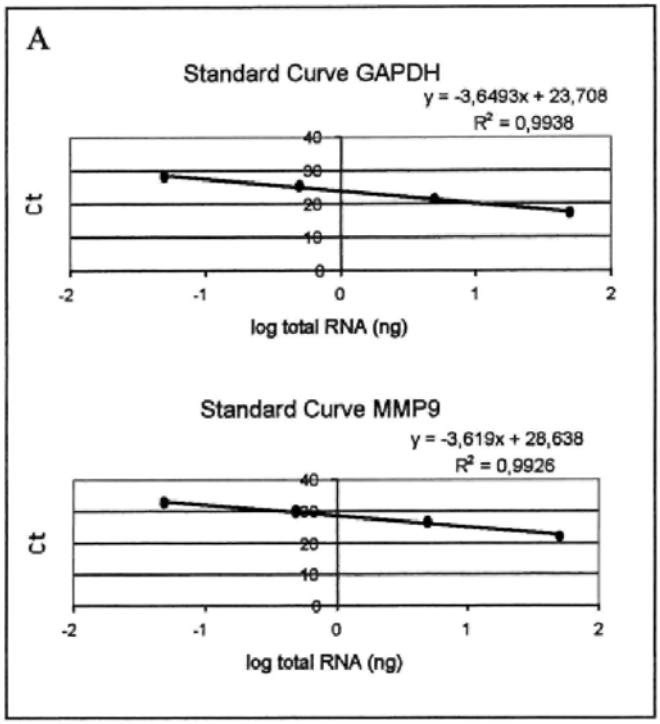
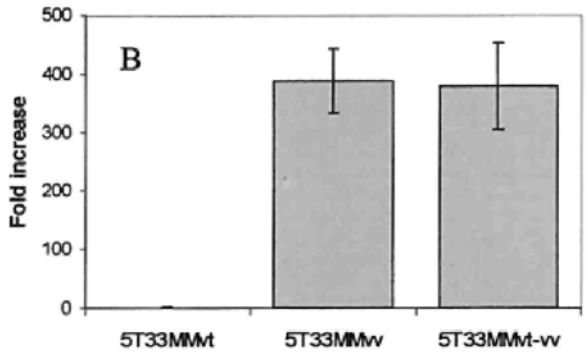
MMP-9 induction is regulated at the transcriptional level

To determine whether the upregulation of MMP-9 was regulated at the transcriptional level, we performed quantitative real-time PCR. Standard curves and line equations are given in Figure 5*a.* The amounts of MMP-9 and GAPDH mRNA for 5T33MMvt, 5T33MMvv, 5T33MMvt-vv and normal BM samples were calculated as described in Material and Methods. These results and the normalized amounts of MMP-9 are given in Table I. The purity of the *in vivo* samples was 94% for 5T33MMvv and 81% for 5T33MMvt-vv. Therefore, we subtracted 6% (0.13) and 19% (0.41) of the normalized MMP-9 amount of normal BM from the normalized amount of 5T33MMvv and 5T33MMvt-vv, respectively. These values were then divided by the normalized MMP-9 amount of 5T33MMvt. As shown in Figure 5*b,* the level of MMP-9 mRNA was higher in the *in vivo* growing cells, 5T33MMVV (388x) and 5T33MMvt-vv (378x) compared to the level in the *in vitro* growing cells, 5T33MMvt (1x). We can thus conclude that induction of MMP-9 is regulated at the transcriptional level.

FIGURE 5 : *(a) Standard curves generated by plotting C_t values vs. log total RNA. Equations and r^2 value are shown, (b) Relative fold induction of MMP-9 mRNA in in vivo samples compared to the 5T33MMvt sample calculated with the standard curve method. Error bars represent SD. Results from 1 representative experiment of 2 are shown.*





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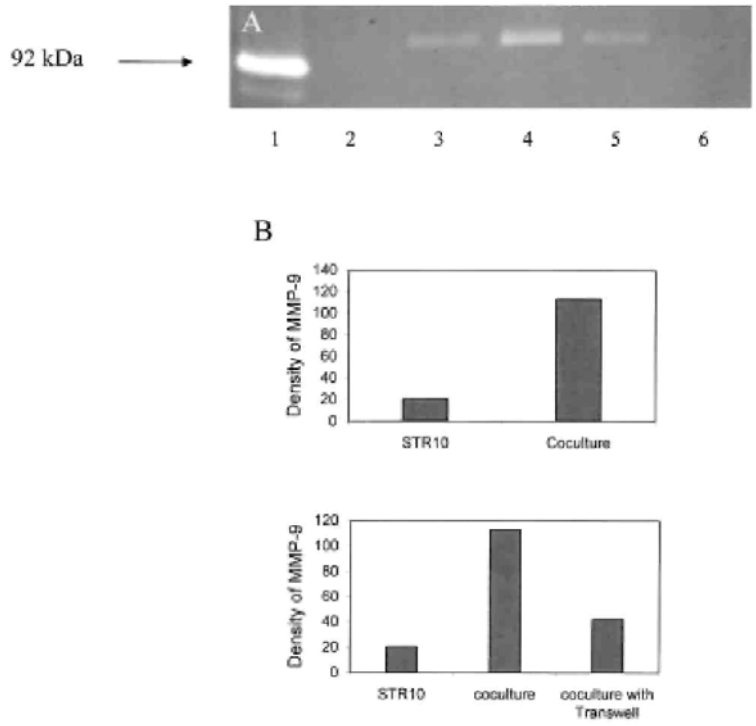
Upregulation of MMP-9 in MM cells after contact with BMECs

The BM microenvironment is a complex structure of various extracellular components and many cell types, including BMECs. BMECs act as gatekeepers, separating the BM compartment from the intravascular compartment, and are the first cells encountered by the MM cells upon entry to the BM environment from the blood circulation. To investigate if the interaction between BMECs and MM cells induces MMP-9, we established cocultures of STR10 BMECs and 5T33MMvt cells and examined MMP-9 expression by zymography. Media derived from the cocultures contained higher levels of MMP-9 compared to media obtained from each cell type cultured separately (Fig. 6*a*). Densitometry of the MMP-9 band in zymography revealed an average 9-fold induction of MMP-9 in the cocultures (Fig. 6*b*, upper, $p < 0.028$). Double FACS staining of the cocultures with MMP-9 and 5T33MM anti-idiotypic antibodies demonstrated that MMP-9 is induced in 5T33MMvt cells (Fig. 7) and not in STR10 cells (data not shown). Cocultures of 5T33MMvt cells with lung endothelial cells (LE1SVO) did not cause induction of MMP-9 in the MM cells (Fig. 8).

TABLE I : *GAPDH AND MMP-9 mRNA AND NORMALIZED MMP-9 AMOUNTS OF THE UNKNOWN SAMPLES IN QUANTITATIVE REAL-TIME PCR*

Sample	Average GAPDH (ng)	Average MMP-9 (ng)	MMP-9 normalized to GAPDH (ng)
5T33vt	162 ± 7.21	0.959 ± 0.272	0.00592 ± 0.002
5T33VV	23.8 ± 1.57	57.9 ± 6.64	2.43 ± 0.32
5T33vt-vv	35.2 ± 1.29	93.3 ± 14.2	2.65 ± 0.41
Normal BM	27.6 ± 1.22	59.0 ± 3.29	2.14 ± 0.15

FIGURE 6 : *(a) Gelatin zymography of conditioned medium of STR10 BMECs alone (lane 3), STR10 cells in coculture with 5T33MMvt cells (lane 4), STR10 cells in coculture with 5T33MMvt cells with a transwell (lane 5) and 5T33MMvt cells alone (lane 6). HT1080 (lane 1) and serum-free medium (lane 2) represent, respectively, positive and negative controls. Results from 1 representative experiment of 3 are shown, (b) Densitometry of the MMP-9 band in gelatin zymography. Upper: Results of densitometry performed on the MMP-9 band in the zymography of the STR10 cells alone and of the coculture (STR10 + 5T33MMvt). Results from 1 representative experiment of 6 are shown. The increased density of MMP-9 in cocultures compared to STR10 alone is statistically significant ($p < 0.028$), as determined with the Wilcoxon signed rank test. Lower: Results of densitometry performed on the MMP-9 band in the zymography of STR10 cells alone, of coculture and of coculture with transwell. Results from 1 representative experiment of 3 are shown.*



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A soluble factor secreted by BMECs is responsible for MMP-9 induction

When cells were cocultured in a transwell separating upper and lower compartments, there was still upregulation of MMP-9 (Fig. *6a)* but to a lesser extent than in cocultures with direct contact (Fig. *6b,* lower). We conclude that the *in vivo* induction of MMP-9 in MM cells is stimulated by a soluble factor produced by BMECs.
We incubated 5T33MMvt cells with SDF-1, IGF-I, IL-6, MCP-1, LN and FN at the concentrations mentioned in Material and Methods and analyzed MMP-9 production by gelatin zymography. None of these factors caused upregulation of MMP-9 (data not shown). Syndecan-1 surface expression was analyzed by FACS staining. There was no difference in the expression of syndecan-1 on myeloma cells before and after coculture (data not shown).

FIGURE 7 : *MMP-9 expression in 5T33MMvt cells before and after coculture with STR10 BMECs. Open histogram shows MMP-9 expression in 5T33MMvt cells before coculture. Shaded histogram shows expression of MMP-9 in 5T33MMvt cells after coculture. Results from 1 representative experiment of 3 are shown.*

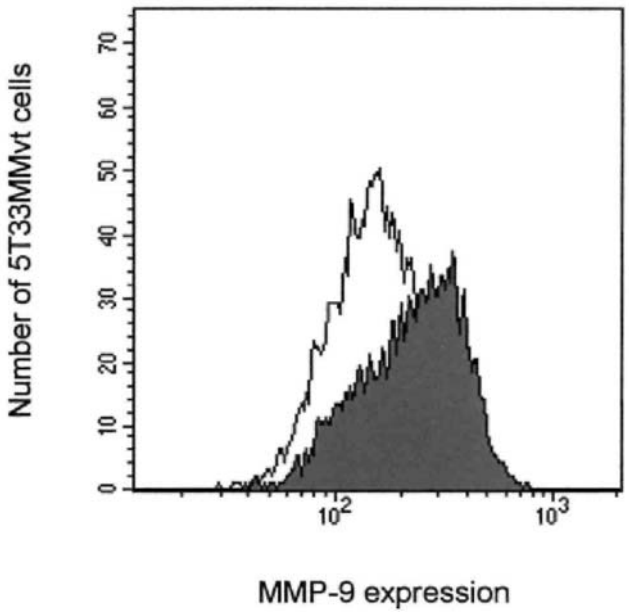
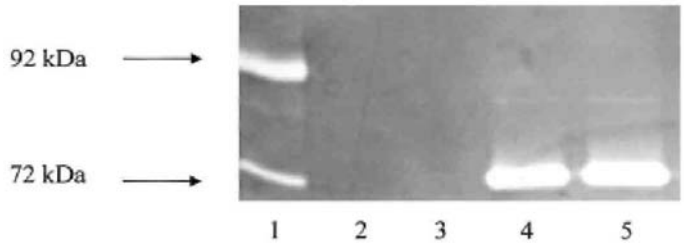


FIGURE 8 : *Gelatin zymography of cocultures between 5T33MMvt cells and lung endothelial cells (LE1SVO) with positive control (lane 1), negative control (lane 2), 5T33MMvt cells alone (lane 3), LE1SVO cells alone (lane 4) and LE1SVO cells in coculture with 5T33MMvt cells (lane 5). Results from 1 representative experiment of 3 are shown.*



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DISCUSSION

Several studies have demonstrated a role of MMPs in cancer. MMP production was demonstrated in MM.[11,14,28] We used the 5T33MM mouse model to investigate the production of MMP-9 (gelatinase B) in MM. Similar to human MM, we demonstrated by gelatin zymography MMP-9 production by 5T33MMvv cells. The 5T33MMvt cell line was generated and proven to be clonally identical to the 5T33MMvv cell line, by Ig sequence analysis.[26] When the MMP-9 production of this cell line was analyzed, no MMP-9 activity was observed. This agrees with the data of Barillé *et al.,*[11] who compared the conditioned media of primary myeloma cells (patient SBN-1) and of the cell line (SBN-1) established from these myeloma cells. The SBN-1 cell line, which was immortalized for almost 1 year, had no detectable MMP-9 activity compared to fresh myeloma cells, which produce a significant amount of MMP-9. To analyze whether the BM microenvironment is involved in the regulation of this differential MMP-9 production, 5T33MMvt tumor cells were injected into naive mice and, upon development of disease, BM (5T33MMvt-vv cells) was isolated and analyzed for MMP-9 production. 5T33MMvt-vv cells secreted a significant amount of MMP-9. These results are comparable to the data on prostate cancer cells, which produced undetectable amounts of pro-MMP-9 *in vitro* but stained positive for MMP-9 when grown in human bone implants.[29] Real-time PCR demonstrated that *in vivo* induction of MMP-9 is regulated at the transcriptional level with 388× and 378× more MMP-9 mRNA in, respectively, 5T33MMVV and 5T33MMvt-vv cells than in 5T33MMvt cells (1x). When 5T33MMvt-vv cells were cultured, the MMP-9 in the conditioned media disappeared completely after 4-10 weeks, suggesting that tumor-stroma interactions play a role in the induction of MMP-9 *in vivo.* MMP-9 has as a major substrate type IV collagen, a structural component of the basement membrane that is involved in transendothelial migration. The first cell type that MM cells come across when they home to the BM is the BMEC. For this reason, we investigated whether the interaction between BMECs and MM cells is important for the *in vivo* upregulation of MMP-9. We established cocultures of STR10 cells with 5T33MMvt cells and analyzed MMP-9 production by gelatin zymography. After 48 hr, we observed a 9-fold induction of MMP-9 in the cocultures, especially in 5T33MMvt cells as measured by double FACS staining. This is similar in T lymphoma, where adhesion of tumor cells with endothelial cells resulted in a significant increase in MMP-9 production.[30] MMP-9 induction in cocultures is cell type-specific since cocultures of 5T33MMvt cells with lung endothelial cells (LE1SVO) demonstrated no upregulation of MMP-9 after 48 hr. These results correspond to previous results of immediate homing of the 5TMM tumor cells, where we could observe only MM cells in the BM and spleen but not in the lungs.[31] All of these data suggest that the interaction of MM cells with endothelial cells is, at least partly, responsible for the *in vivo* upregulation of MMP-9 in MM cells and that this event is specific for BMECs. The interaction of BMECs with MM cells can be divided into 2 different types: a soluble interaction and a direct cell contact interaction. Cocultures with a transwell to prevent direct cellular contact also demonstrated upregulation of MMP-9, though less than when contact was allowed, suggesting a role of a soluble factor secreted by BMECs in the *in vivo* upregulation of MMP-9. Many factors are described as stimulators for MMP-9. SDF-1 induced migration of megakaryocytes by increasing MMP-9 production.[32] Mira *et al.*[33] showed that MMP-9 secretion is induced when MCF-7 human breast carcinoma cells were treated with IGF-I. IGF-I is a chemoattractant for 5T2MM cells.[34] Syndecan-1 is an important regulator in MM, which inhibits invasion of myeloma cells, mediates cell-cell adhesion and regulates cell growth.[10] Expression of syndecan-1 on myeloma cells suppresses the level of MMP-9 in the conditioned medium of tumor cells.[35] Our group previously showed that MCP-1 is expressed by BMECs and is chemotactic for 5TMM cells.[36] Stuve *et al.*[37] demonstrated that MCP-1 increased MMP-9 secretion by PBMCs and that the MCP-1-enhanced migration of PBMCs is blocked by TIMP-1. IL-6, a cytokine involved in MM, has also been described to induce MMP-9 activity.[38] There is evidence that ECM components can regulate MMP secretion. Treatment of ovarian cancer cells with FN enhanced MMP-9 production.[39] Macrophages incubated with LN showed increased secretion of MMP-9.[40] None of these factors (SDF-1, IGF-I, MCP-1, IL-6, FN and LN) caused upregulation of MMP-9 in 5T33MMvt cells. There was no difference in the expression of syndecan-1 on myeloma cells before and after coculture. Further studies are required to identify the soluble factor involved in the upregulation of MMP-9 described here.

There is evidence that interactions between MM cells and the BM microenvironment contribute to tumor cell survival and tumor growth.[41] IL-6, the most important growth factor in MM, is upregulated when myeloma cells bind to BMSCs.[42] The tumor microenvironment prevents CD95-induced apoptosis in myeloma cells[43] as well as chemotherapy-induced cell death in myeloma cell lines.[44] These results suggest that targeting the interaction between tumor cells and the microenvironment could represent an interesting therapeutic approach in MM. In the present work, we demonstrate the importance of the interactions between MM cells and BMECs in the regulation of MMP-9 production. According to the model of Butcher and Picker,[45] migration of lymphocytes through the endothelium is a multistep cascade of processes, among which transient adhesion of the cells to endothelial cells is followed by stable arrest. Several adhesion molecules mediate this process. Inhibiting this interaction and, thus, the arrest of MM cells in the bloodstream of BM sinusoids would prevent MMP-9 upregulation. Disruption

of this interaction between MM cells and BMECs would be important not only for preventing tumor spread but also for preventing MM-associated bone destruction and angiogenesis.

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