

Bone marrow endothelial cells increase the invasiveness of human multiple myeloma cells through upregulation of MMP-9: evidence for a role of hepatocyte growth factor

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The migration of multiple myeloma (MM) cells from the circulation into the bone marrow (BM) implicates that they must have the capacity to cross the BM endothelium including the subendothelial basement membrane. In this study, human CD138⁺ MM cells were immunomagnetically isolated from BM samples of MM patients and their invasion through Matrigel, that is, a reconstituted basement membrane, was determined. We demonstrated that primary MM cells have the capacity to transmigrate through basement membrane and that this invasiveness was considerably increased when assessed on Matrigel filters coated with BM endothelial cells (EC) (4LHBMCE line) (transendothelial invasion). The isolated MM cells were shown by zymography to secrete matrix metalloproteinase (MMP)-9 and anti-MMP-9 antibodies inhibited transendothelial invasion, indicating that MMP-9 is involved in this process. BM EC were found to increase the MMP-9 secretion in MM cells, indicating that EC enhance MM cell invasion through stimulation of MMP-9 secretion. BM EC were found to produce hepatocyte growth factor (HGF), and this cytokine also stimulated MMP-9 secretion in MM cells, while anti-HGF antibodies significantly inhibited EC-stimulated MM cell invasion. In summary, our findings provide evidence that MM cell–BM EC interactions enhance the invasion of human MM cells through stimulation of MMP-9 secretion.

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Introduction

Multiple myeloma (MM) represents a B-cell malignancy characterised by a monoclonal expansion of malignant plasma cells. During the main course of disease evolution, MM cells are predominantly localized within the bone marrow (BM) micro-environment.¹ The migration of MM cells from the circulation into the BM implicates that these cells must have the capacity to cross the BM endothelium. In analogy to the homing and trafficking of normal lymphocytes, one can hypothesize that the BM homing of MM cells is mediated by a multistep process. In a model described by Butcher and Picker,² the trafficking and homing of lymphocytes is represented by an integrated process of leukocyte rolling on the blood vessel wall, followed by activation, firm adherence, and transmigration into tissue.² The last step in the transmigration through the vessel wall requires the degradation of the subendothelial basement membrane, a specialized extracellular matrix structure composed essentially of collagen type IV, laminin, and perlecan.³ It has been proposed that lymphocytes and other leukocytes need to

degrade blood vessel basement membranes to complete diapedesis. It has been demonstrated that matrix metalloproteinases (MMPs), especially gelatinases, are essential in this process.^{4–6}

The MMPs represent a family of zinc-containing proteolytic enzymes.^{7,8} They have been classified according to their substrate specificity into gelatinases, stromelysins, and collagenases. MMPs participate in normal processes; however, they have been associated with advanced-stage cancer and contribute to tumour progression, invasion and metastasis.^{9–11}

A subgroup of the MMP family, the gelatinases or type IV collagenases, degrade native collagen type IV, a major component of the basement membrane, and therefore these proteases are believed to play a crucial role in processes requiring basement membrane degradation, like lymphocyte trafficking.^{7,12} Two gelatinases, MMP-2 or gelatinase A (72 000 type IV collagenase) and MMP-9 (gelatinase B or 92 000 kDa type IV collagenase) have been identified to date.

Previous studies demonstrated the constitutive secretion of MMP-9 by murine MM cell lines, human MM cell lines (8226 and SBN-1) and primary MM cells.^{13,14} However, the exact role of MMP-9 in the pathogenesis of MM has to be elucidated.

Aberrant expression of MMPs is mediated at least in part by soluble factors like cytokines, chemokines, and growth factors, which can therefore influence the invasive behaviour of malignant cells. Stroma–tumour cell interactions may participate in this process by either mediating or augmenting tumour invasion through MMP-9 regulation.^{15–17}

One of the growth factors that have been reported to enhance invasiveness *in vitro* of several cancer cell types associated with increased production of proteases is hepatocyte growth factor (HGF).^{18–21} HGF, also known as scatter factor (SF) is a mesenchymal-derived heparin-binding polypeptide first identified as a potent mitogen for adult rat hepatocytes.²² It is a multifunctional cytokine with pleiotropic effects on a variety of cells involved in biological activities including mitogenesis, morphogenesis, tumour progression and angiogenesis.^{23,24} The biological effects of HGF are mediated through its specific transmembrane receptor, c-Met, a tyrosine kinase.²⁵ Several investigators have suggested that aberrant HGF-c-Met signalling very likely contributes to the growth and progression of several types of neoplasms.^{26–28} Human MM cells were previously shown to express the HGF receptor c-Met.^{29,30} In addition, elevated serum concentrations of HGF were found in patients with MM and were associated with an unfavourable prognosis.³¹ Several possible roles of HGF in the pathogenesis of MM have been suggested including bone destruction and angiogenesis but the exact role is unknown.^{32,33}

The aims of the present study were therefore to investigate (1) whether MM cells are invasive by analysing the capacity to transmigrate through Matrigel, a reconstituted basement membrane, (2) whether endothelial cells (EC)–MM cell interactions modulate the invasiveness of MM cells and (3) whether MMP-9 is involved in MM cell invasion.

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In this study, we demonstrated that human MM cells have the capacity to transmigrate through reconstituted basement membrane, a process that is enhanced by BM EC through stimulation of MMP-9 secretion. We provide also evidence that HGF is involved in this EC-mediated stimulation of MMP-9 secretion by the MM cells, and therefore potentially contributes to the extravasation and BM homing of MM cells.

Materials and methods

Patient samples and isolation of primary MM cells

BM samples from eight MM patients (pts 1–8) were collected during standard diagnostic procedures. Each MM patient was diagnosed and staged according to the criteria of Durie and Salmon.³⁴ The local ethical committee approved the study. BM aspirates were obtained from the posterior iliac crest or sternum, and collected in a heparinised syringe. Mononuclear cells (MNC) were separated by Ficoll density gradient centrifugation (Nycomed, Lucron, Gent, Belgium). Primary MM cells were immunomagnetically separated using the Magnetic Cell Sorting System (MACS) (Miltenyi Biotech, Bouchout, Belgium). MNC were incubated for 15 min at 4°C with MACS microbeads conjugated to a monoclonal mouse CD138 (syndecan-1) antibody (clone B-B4, isotype mouse IgG1) (40 µl/10⁷ cells). Cells were washed once in phosphate-buffered saline (PBS) supplemented with human albumin (4%), resuspended, and separated on a column placed in the magnetic field of the MACS separator. CD138+ cells were retained and eluted as a positively selected cell fraction after removal of the column from the magnetic field. Cells were counted and viability was assessed with trypan blue. MACS purification revealed a 97–99% pure primary MM cell population as determined after May–Grünwald–Giemsa-staining of a cytospin preparation.

BM EC

The BM EC line 4LHBMEC (kindly provided by Dr A Dräger, Department of Hematology, Free University Amsterdam, The Netherlands) was cultured in 10% FCS-199 medium (International Medical, Brussels, Belgium) with 10 ng/ml ECGF (Roche, Brussel, Belgium) at 37°C and 5% CO₂ until a confluent adherent cell layer was obtained. Cells were recovered for further use after trypsinisation.

Preparation of MM cell-conditioned media

Isolated primary MM cells (10⁶) from MM patients were washed, resuspended in serum-free RPMI medium (5 ml) (Invitrogen, Merelbeke, Belgium) and incubated for 48 h (at 37°C and 5% CO₂) in serum-free RPMI medium. MM cell-conditioned medium (MM CM) was collected, centrifuged to remove cell debris, concentrated 25-fold using Vivapore devices (Vivascience, Vilvoorde, Belgium), and stored at –20°C until use. In some experiments, MM CM was prepared after 48 h incubation in the presence of recombinant HGF (100 ng/ml) (Sigma, Bornem, Belgium).

Coculture of MM cells with BM EC

MM cells (1 × 10⁶) were added to confluent monolayers of BM EC in six-well plates in serum-free RPMI (5 ml). After 48 h of

incubation at 37°C and 5% CO₂, culture supernatant was harvested, concentrated 25-fold and used for zymography.

Gelatin zymography

Gelatinase activity present in CM obtained from MM cells was assessed under nonreducing conditions using a modified sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (zymography).³⁵ Amounts of 10 µl of CM mixed with 10 µl of loading buffer (Novex[®] Tris-Glycine SDS sample buffer (2 ×) (Invitrogen) were subjected to electrophoresis using the Novex[®] precast 10% gelatin zymogram gels (Invitrogen). Electrophoresis was performed using the Xcell *sureLock*[™] electrophoresis apparatus (Invitrogen) under constant voltage (150 V) for 2–4 h. After electrophoresis, gels were washed with the Novex[®] zymogram renaturing buffer (Invitrogen) for 30 min to remove SDS and incubated at 37°C for 20 h in the Novex[®] zymogram developing buffer (Invitrogen). The gels were then stained with 0.05% Serva Blue R stain (Invitrogen) in 10% ethanol and 10% acetic acid, and destained with 10% ethanol and 10% acetic acid. The proteolytic activity was identified as clear white bands against a blue background staining. CM from the HT1080 fibrosarcoma cell line was used as a positive control for MMP-9 secretion.³⁶ After destaining, zymograms were dried (DryEase[®] Mini-Gel Drying System), and the intensity of the gelatinolytic bands was quantified using NIH image 1.58 for Windows software.

Matrigel invasion assay

The invasiveness of MM cells was analysed using invasion assays with BioCoat Matrigel invasion chambers (24-well) (Becton Dickinson, Erembodegem, Belgium). In this assay, invasion was measured by determining the ability of MM cells to migrate through Matrigel, a reconstituted basement membrane.^{37,38} A quantity of 300 µl RPMI 1640 with 20% FCS, which served as chemoattractant, was added to the lower compartment of the invasion chamber. In some experiments, BM EC CM was added to the lower compartment. The lower compartment was overlaid with an 8-µm pore size polyethylene (PET) membrane, precoated with Matrigel basement membrane matrix. Primary MM cells (10⁵) suspended in 100 µl serum-free RPMI 1640 were seeded in the upper compartment of the prehydrated Matrigel-coated invasion chambers and incubated for 18 h. Cells that invaded the Matrigel-coated filters were recovered from the lower compartments and counted by FACSsort flow cytometer (Becton Dickinson). A known number of sphero blank calibration beads (Becton Dickinson) were added as an internal standard for quantification of the migrated cells. The migrated MM cells were discriminated from potentially migrated EC by their different forward scatter/sideward scatter characteristics.

To examine the role of MMP-9 and HGF in transendothelial invasion, MM cells together with anti-MMP-9 monoclonal antibodies (MoAb) (IgG1) (10 µg/ml) (Calbiochem, VWR, Leuven, Belgium), anti-HGF MoAb (IgG₁) (10 µg/ml) (Sigma), or control antibody were loaded into the upper compartments of the BioCoat Matrigel invasion chambers, and the invasion assay was performed as described above.

The results were represented as the invasion index, which was calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment. For invasion-inhibition experiments, results

were presented as the percentage cell invasion compared with control invasion in the absence of MoAb. Each assay was carried out in triplicate.

Transendothelial invasion assay

To assess the effect of MM cell-BM EC interactions on MM cell invasiveness, invasion was evaluated in the presence of a BM endothelial cell layer. For transendothelial invasion assays, BM EC cells (1×10^4) were plated onto Matrigel-coated PET membranes (pore size $8 \mu\text{m}$) in the upper cell culture insert chamber and cultured until confluence (48 h). MM cells (1×10^5 in $100 \mu\text{l}$ RPMI) were then seeded on the BM EC and allowed to invade for 18 h as described for the Matrigel invasion assay. At the end of incubation, cells that migrated through BM EC monolayers and Matrigel were recovered from the lower compartment and counted by FACS.

FACS analysis of *c-Met* expression

The expression of *c-Met* on MM cells was previously demonstrated.²⁹ *c-Met* expression on primary MM cells from the eight MM patients included in our study was confirmed by flow cytometric analysis. *c-Met* was detected by a double-staining procedure. MNC, isolated from BM samples by Ficoll gradient centrifugation, were incubated with an anti-*c-Met* MoAb (IgG_{2a}) (clone 95309) (R&D) (Abingdon, UK) or control mouse IgG_{2a} for 30 min at 4°C and a Cy-5 conjugated CD38-specific antibody (clone HIT2) (Becton Dickinson). In the second step, cells were incubated with PE-conjugated goat anti-mouse IgG_{2a} antiserum (Southern Biotechnology Associates Inc., Birmingham, AL, USA). After the final wash, cells were subjected to FACS analysis (EPICS XL flow cytometer). The presence of monoclonal plasma among the CD38+ gated cells was checked by intracytoplasmic κ/λ immunofluorescence using a three colour staining (human Ig κ FITC and Ig λ -PE (Fab)2 fragments (both from Dako diagnostics, Heverlee, Belgium).

Enzyme-linked immunosorbent assay for HGF

HGF production of BM EC was analysed in CM with a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Biosource, Nivelles, Belgium). The lower limit of detection was 10 pg/ml.

Results

Invasive activity of human MM cells

Since it can be assumed that MM cells need to penetrate actively through the subendothelial basement membrane of the BM sinus to migrate into the BM cavity, the invasive properties of human MM cells were evaluated using the Matrigel invasion assays. In this assay, invasion was measured by determining the ability of MM cells to transmigrate through Matrigel, a reconstituted basement membrane. As shown in Figure 1, primary MM cells isolated from BM samples of eight MM patients showed invasive activity with a range of invaded cells between 2 and 12%.

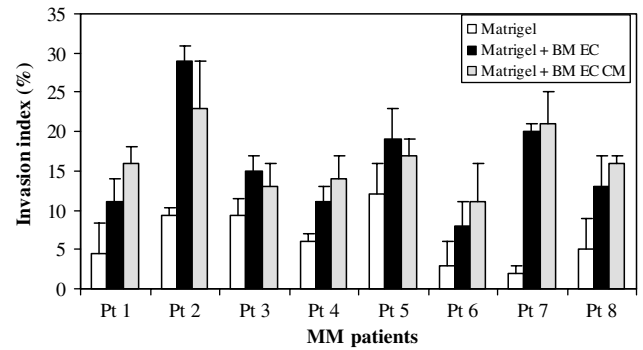


Figure 1 Matrigel invasion of immunomagnetically isolated primary MM CD138+ cells was tested using Biocoat Matrigel invasion chambers. RPMI with 20% FCS was used as chemoattractant. For transendothelial invasion assay, MM cells were allowed to transmigrate for 18 h in Biocoat Matrigel Invasion Chambers in which inserts were coated with a BM EC monolayer, as indicated. Invasion assays were also performed with BM EC CM added to the lower compartment of the Biocoat Matrigel invasion chamber. Matrigel invasion assays were performed with MM cells from eight patients (pts 1–8) and for each assay the experiment was carried out in triplicate. The number of invaded cells was quantified by flow cytometry. The results are expressed as the invasion index (percentage of invading cells over the total number of input cells) (mean value \pm standard deviation (s.d.)).

BM EC enhance penetration of MM cells across reconstituted basement membrane

As host–tumour interactions can stimulate tumour cell invasion, we have performed Matrigel invasion assays using Biocoat Matrigel Invasion Chambers in which inserts were coated with BM EC. Under these conditions, cell invasion with MM cells from eight MM patients was markedly enhanced with a mean value of 16% (range 8–29%) of cells transmigrating through BM EC and Matrigel into the lower chambers, compared with control inserts just containing Matrigel. This indicates that BM EC can stimulate the invasive behaviour of human MM cells (Figure 1). Next it was determined if cell–cell contact between the MM cells and BM EC was required in this process. Therefore, we performed Matrigel invasion assays in the presence of BM EC CM in the lower chambers. Quantitation of the number of invaded cells revealed that 11–23% of MM cells had invaded Matrigel, indicating that MM cell invasion is stimulated by (a) soluble factor(s) secreted by the BM EC (Figure 1).

MMP-9 secretion by human MM cells

We have studied MMP-9 secretion in CM of primary CD138+ MM cells from eight different patients. Zymographic analysis on gelatin-containing gels with serum-free MM CM and CM from HT1080 fibrosarcoma cells (positive control) showed bands of gelatinolytic activity corresponding to MMP-9 (92 kDa gelatinase) in all samples tested (Figure 2).

EC-induced MM cell invasion is dependent on metalloprotease activity

To test the hypothesis that the invasive ability of MM cells is related to the proteolytic activity of MMP-9, we performed

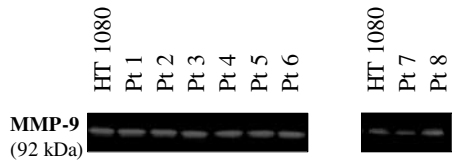


Figure 2 Gelatin zymography for MMP-9 activity in MM CM. Serum-free CM from primary MM cells from eight patient samples was harvested, concentrated and subjected to gelatin zymography. Gelatinolytic activity is indicated by clear zones of gelatin lysis against a dark background of stained substrate. CM from HT1080 fibrosarcoma cells was used as a positive control. Gelatin zymographic analysis was performed three times for each CM sample. Representative gelatin zymograms are shown.

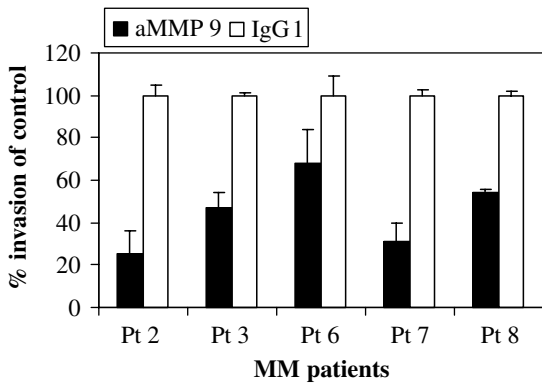


Figure 3 Effect of anti-MMP-9 MoAb on the transendothelial invasion of primary MM Cells. For invasion-inhibition experiments, MM cells were incubated with MMP-9 MoAb (10 μ g/ml) as described or a control antibody (IgG1) (10 μ g/ml) prior to the invasion assay. All experiments were performed in triplicate and results indicate the percentage invasion compared to control invasion in the absence of MoAb (mean value \pm s.d.).

transendothelial invasion assays in which MM cells were incubated with anti-MMP-9 MoAb (10 μ g/ml) during the invasion assay. This experiment was performed using CD138+ MM cells from five different patients (pts 2, 3, 6, 7 and 8). As shown in Figure 3, anti-MMP-9 MoAb significantly inhibited MM cells, to invade the Matrigel barrier by about 55% (range 32–75%) as compared with the invasion in the absence of MoAb or in the presence of irrelevant IgG₁ isotype antibody, suggesting the involvement of MMP-9 in MM cell invasion.

BM EC mediate MM cell invasion by increasing MMP-9 secretion

The relation between MM cell invasion and MMP-9 secretion was further explored by analysing the effect of BM EC–MM cell interactions on the secretion of MMP-9 by the MM cells. MMP-9 release in supernatants of coculture of BM EC and MM cells was investigated for four different MM patients (pts 2, 3, 7 and 8). Elevated levels of MMP-9 (1.7–5-fold increase) were observed as detected by gelatin zymography (Figure 4). This effect could also be induced when MM cells were incubated with CM from BM EC (data not shown), indicating that factor(s) secreted into CM from BM EC enhanced the release of MMP-9 from the MM cells.

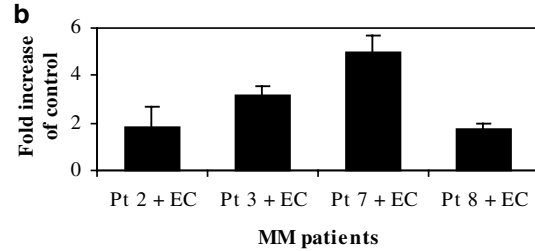
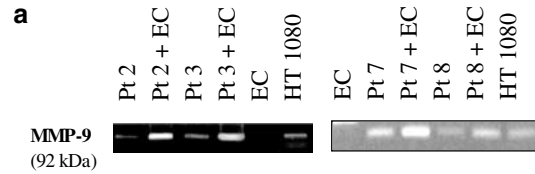


Figure 4 (a) Effect of BM EC on MMP-9 secretion. Gelatin zymography of coculture CM, showing increasing levels of MMP-9 secretion following coculture with BM EC. MMP-9 activity in BM EC CM was also assessed. Zymograms representative of three independent experiments with primary MM cells from four patients (pts 2, 3, 7 and 8) are shown. (b) Densitometric analysis of MMP-9 secretion in MM CM. The results represent the fold increase in MMP-9 activity compared to control (mean \pm s.d. for three analyses).

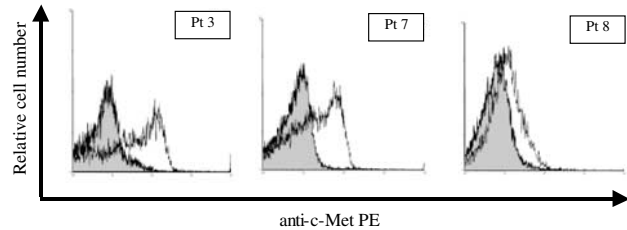


Figure 5 Flow cytometric analysis of c-Met expression on primary MM cells. Results are shown as fluorescence histograms (open histogram: c-Met expression; filled histogram: isotype matched control antibody). Representative histograms from three MM patients are shown (pts 3, 7 and 8).

HGF is secreted by BM EC and stimulates MMP-9 secretion of c-Met-expressing MM cells

To further explore the mechanisms responsible for the increased invasiveness of MM cells through BM EC, we quantified HGF secretion by BM EC using ELISA. HGF levels could be detected in CM samples from BM EC (mean concentration 2.11 ng/ml for five independent measurements) (data not shown). C-Met expression by primary MM cells was analysed by FACS. Flow cytometry with anti-c-Met MoAb demonstrated surface expression of c-Met on primary MM cells in BM samples from eight MM patients tested. FACS profiles of c-Met expression in primary MM cells from three representative BM samples (pts 3, 7 and 8) are shown in Figure 5. Subsequently, we determined whether HGF also stimulates MMP-9 secretion by MM cells. Therefore, MM cells isolated from four patients (pts 1, 6, 7 and 8) were incubated with recombinant HGF (100 ng/ml) for 48 h and MMP-9 secretion in MM CM was determined by gelatin zymography. A 2.3–4.3-fold increase in MMP-9 secretion was observed in CM from MM cells incubated with HGF for 48 h when compared with controls without exposure to HGF (Figure 6).

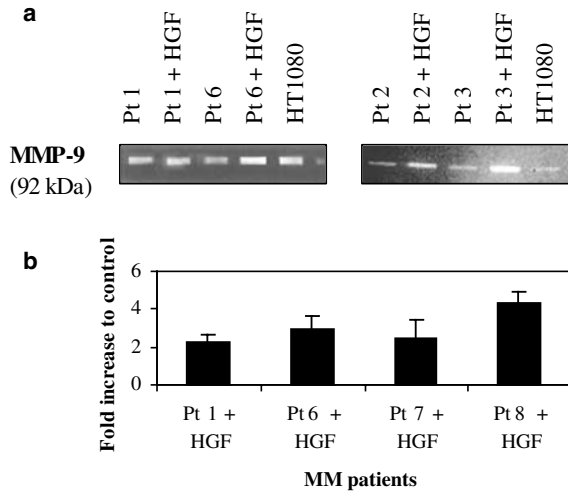


Figure 6 Effect of HGF on MMP-9 secretion: (a) CM from MM cells incubated with HGF (100 ng/ml) for 48 h was subjected to zymographic analysis and MMP-9 activity after HGF incubation was compared to control without incubation. Gelatin zymography of serum-free CM of primary MM cells from four patients is shown (pts 1, 6, 7 and 8); (b) Quantification of the gelatinolytic activity detected in MM CM by densitometry. The results represent the fold increase in MMP-9 activity compared to control without incubation with HGF. Columns represent the mean fold increase of density \pm s.d.

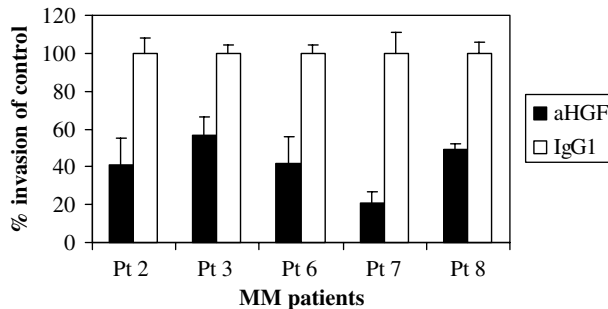


Figure 7 Effect of HGF MoAb on the transendothelial invasion of MM cells. MM cells were incubated with HGF MoAb (10 μ g/ml) or an irrelevant mouse IgG1 isotype antibody as described prior to the transendothelial invasion assay. Assays were performed in triplicate for five patients (pts 2, 3, 6, 7 and 8). The results are presented as the percentage invasion compared to control in the absence of MoAb (mean value \pm s.d.).

HGF is involved in MM cell transendothelial invasion

To investigate the role of HGF in regulating MM cell invasion, MM cells from five patients (pts 2, 3, 6, 7 and 8) were incubated with anti-HGF MoAb (10 μ g/ml) prior to the transendothelial invasion assay. As shown in Figure 7, transendothelial invasion was significantly inhibited by anti-HGF MoAb (43–79% inhibition), whereas irrelevant mouse IgG₁ did not affect MM cell invasion. These results suggest that HGF is involved in MM cell invasion, by modulating gelatinase secretion.

Discussion

Despite significant advance in the understanding of the biology of MM, the molecular mechanisms mediating the selective

localisation of MM cells in the BM are not fully understood. Previously, our group has demonstrated in the 5T2 MM murine model that the specific localisation of MM cells in the BM is due to a combination of selective BM homing and survival in the BM micro-environment.³⁹ Based on a model of Butcher and Picker,² describing lymphocyte homing and trafficking, the migration of MM cells from the circulation into the BM compartment is likely to occur as a multistep process of rolling, firm adhesion, transmigration through the endothelium and the subendothelial basement membrane and finally, migration to the BM stroma, influenced by chemotactic factors.² We have previously demonstrated that the monocyte chemotactic proteins (MCP-1, -2 and -3), chemokines produced by the BM stroma, act as potent chemoattractants of MM cells through binding to CCR2, indicating a potential contribution of these chemokines to MM cell homing.⁴⁰ Besides specific migration influenced by locally produced chemoattractants, the homing of MM cells also depends on transmigration through the BM endothelium and the subendothelial basement membrane. In a previous study, we have demonstrated that MM cells express the high-affinity laminin binding protein 67LR, which mediates MM cell migration to laminin-1, a major component of the basement membrane. 67LR expression on MM cells was found to increase by contact with the endothelium, indicating that during passage through the endothelium, 67LR is contemporary upregulated allowing MM cells to become responsive to the chemoattractive properties of laminin-1, and therefore facilitating transendothelial migration.⁴¹ The final step in the extravasation process from the circulatory system to the extravascular tissue requires penetration of the vessel wall involving migration through the endothelium and passage through the subendothelial basement membrane. This process requires degradation of the basement membrane, which involves the activation of MMPs, a family of zinc-dependent proteases that regulate proteolysis of most ECM components. Among these MMPs, MMP-9 that preferentially degrades type IV collagen, has been largely implicated in basement membrane degradation associated with tumour invasion. MMP-9 overexpression has indeed been correlated with an invasive phenotype in various cancer cell types.^{9,10} Moreover, it has been proposed that during trafficking and homing, also normal leukocytes need to degrade blood vessel basement membrane components to complete diapedesis and the involvement of MMP-9 in this process has been demonstrated.^{5,6} In previous reports, a constitutive production of MMP-9 by MM cells (murine MM cells, MM cell lines and primary MM cells) was demonstrated.^{13,14} Moreover, MMP-9 appeared to be involved in the invasion of 5T2 MM and 5T33 MM cells.⁴² Recently, our group has shown that EC can upregulate MMP-9 secretion in MM cells in the 5 T murine MM model.¹⁴ However, it is not clear yet as to which factor produced by the EC is responsible for this upregulation, and whether a similar effect is also inducible in human MM cells.

In the present study, we used freshly isolated human CD138+ MM cells and BM EC to investigate the ability of MM cells to transmigrate through reconstituted basement membrane (Matrigel) and to determine the role of MMP-9 in this process.

Using Biocoat Matrigel invasion chambers, we found that MM cells are indeed invasive and that BM EC could considerably enhance their invasion capacity. Our results indicate that MM cell-EC interactions facilitate the ability of MM cells to transmigrate through Matrigel. We could confirm that human MM cells secrete bioactive MMP-9 and provide evidence that this protease is directly involved in the transendothelial invasion of MM cells. Several studies have reported that tumour-host

interactions can mediate tumour cell invasion by stimulating MMP production by the tumour cell.^{16,17} Subsequently, we analysed the effect of BM EC on MM cell MMP-9 secretion. BM EC and MM cells were cocultured and MMP-9 secretion in the coculture supernatants was analysed by gelatin zymography. MM cells secreted a significant larger amount of MMP-9 when cocultured with BM EC. These data suggest that BM EC modulate the invasiveness of MM cells by enhancing MMP-9 activity. MMP-9 secretion was also increased when MM cells were incubated with BM EC conditioned medium, indicating that this stimulation is not mediated by direct cell-to-cell contact but rather involves one or more humoral factor(s) produced by BM EC. Previous studies indicated that MMP-9 secretion could be influenced by several growth factors, including HGF. This cytokine has been shown to stimulate MMP-9 secretion in various cancer cell types. In addition, HGF is an important mediator of tumour-stromal interactions through enhancing cell motility and tumour invasion.^{19,20} It has also been reported that various EC types produce HGF.⁴³ Accordingly, we found that the 4LHBMEC cell line, used as source of human BM EC in this study, also produces HGF. Previous studies indicated that human MM cell lines as well as primary tumour cells express c-Met, the HGF receptor,²⁹ while HGF has been shown very recently to stimulate proliferation and to prevent apoptosis of primary human MM cells.⁴⁴ In this study, we demonstrated that this cytokine can also enhance the production of MMP-9 in human MM cells. Moreover, we found that neutralising antibodies against HGF inhibit *in vitro* the transendothelial invasion of human MM cells. These observations suggest that HGF produced by BM EC triggers transendothelial migration of MM cells by enhancing endogenous MMP-9 production. Some studies revealed that human MM cell lines as well as primary MM cells not only express c-Met but produce, although at very variable levels, also HGF, indicating the possibility of autocrine stimulation.^{30,44} Therefore, it cannot be excluded that HGF-induced stimulation of MMP-9 production in MM cells is not only mediated by BM EC but includes also an autocrine component.

In conclusion, our data indicate that primary human CD138+ MM cells have the capacity to migrate through BM endothelium, including the basement membrane, a process that is stimulated by BM EC. Moreover, we provide evidence that this transendothelial invasion is not only regulated by MMP-9 but involves also HGF, a cytokine that is produced by BM EC and that can directly upregulate MMP-9 production in the tumour cells. It can be assumed that a similar paracrine mechanism is also involved in the extravasation and homing of MM cells *in vivo*. Targeting these molecules might therefore provide new strategies for the treatment of MM.

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