Breast Tumor Cells Transendothelial Migration Induces Endothelial Cell Anoikis Through Extracellular Matrix Degradation

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Abstract. *Mutual interactions between human breast cancer cells and endothelial cells were studied in a model mimicking tumor cell intravasation. MDA-MB-231 tumor cells and human umbilical vein endothelial cells (HUVEC) were cocultured on opposite sides of a Transwell filter allowing tumor cell contacts with the basement membrane of the HUVEC forming endothelium and tumor cell transendothelial migration. Confocal microscopy analysis showed that transmigrating MDA-MB-231 cells lay under the HUVEC, thereby inducing HUVEC detachment and tumor cell-HUVEC contact-dependent apoptosis. GM6001 a matrix metalloproteinase (MMP) inhibitor inhibited almost completely, the MDA-MB-231 cell transendothelial migration and the anoikis process. In this intravasation model, a tumor cell invasive mechanism was demonstrated (i) induction of extensive endothelial anoikis induced by degradation of the extracellular matrix (ECM) components, (ii) activation of pro-matrix metalloproteinase (MMP)-2 into MMP-2 by the MT1-MMP-TIMP (tissue inhibitor metalloproteinase) 2-pro-MMP-2 membrane complex and (iii) attraction and migration of metastatic cell through apoptotic endothelium. These interactions could partly explain the necrosis-angiogenesis relationship in tumor angiogenesis.*

The mammary epithelium and stroma interact during the normal development and function of the gland but these interactions are altered during tumor development (1). Tumor angiogenesis is a complex multistep process driven by local positive and negative signals within the tumor. This involves the degradation of the extracellular matrix (ECM) around a local venule, the proliferation and migration of capillary endothelial cells and their differentiation into functioning capillaries (2). Tumor-associated neovascularization is a prerequisite of rapid tumor development and subsequent metastasis formation in distant organs (2). A statistically significant correlation has been found between metastasis incidence and microvessel formation in invasive breast tumors (3). We have reported previously that autocrine and paracrine regulation by angiogenic growth factors is directly involved in the progression of *in vitro* and *in vivo* experimental tumors (4-7). However, we did not study the direct interactions between tumor and endothelial cells. The mechanism by which tumor cells, present in the blood circulation, cross the endothelial barrier to reach the extravascular space (extravasation) was well documented. Thus previous studies have shown that, in extravasation models, breast tumor cells alter the endothelium integrity by inducing endothelial cell apoptosis, whereas in contrast, normal epithelial cells do not seem to damage the endothelium (8). However, the first step of tumor cell invasiveness, *i.e.* the intravasation of tumor cells through the basal face of the stromal vasculature, requires mutual interactions between tumor and endothelial cells and remains poorly understood. In order to investigate the mutual interactions between breast tumor cells and endothelial cells during tumor development and metastasis formation, we designed a new *in vitro* intravasation model that mimics *in vivo* intravasation as it allows direct contact between tumor cells, basement membrane and basal pole of endothelial cells, human umbilical vein endothelial cells (HUVEC). A

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primary culture of HUVEC by forming a confluent cell monolayer on the lower side of a Transwell filter are representative of a vessel endothelium, and tumor cells cultured on the upper side can migrate through the pores of the filter and the basement membrane of the HUVEC endothelium.

Gelatinases have been shown to play a role in the invasiveness of both normal and neoplastic cells by mediating the degradation of the ECM. Several studies provide evidence of the cell surface association and regulation of several members of the matrix metalloproteinase (MMP) family including the MMP-2 gelatinase and the membrane type MMPs (10). Thus, high-affinity binding site for MMP-2 has been observed in MCF-7 and MDA-MB-231 breast cancer cells (9) and activation of latent MMP-2 (pro-MMP-2) and the metastatic capacity of invasive cancer cells has been shown both *in vitro* and *in vivo* (10). Gelatinases can also degradade the basement membrane of vessels leading to an increased metastatic process. Only, the active form of MMP-2 is involved in invasivion and metastasis (10). The interaction of MMP-2 with surface-associated laminin, collagen and fibronectin has been demonstrated (10). Moreover laminin and collagen IV have been shown to be necessary for endothelial differentiation in whole vascular tubes on Matrigel (11). Thus laminin deletion blocked capillary maturation in mice (11) and MMP-2 increased the migration of breast cancer tumors cells on laminin by degradading this ECM component (12).

In this study the transendothelial migration of MDA-MB-231 and MCF-7 tumor cell lines was compared. The MDA-MB-231 cells, isolated from a pleural effusion of breast adenocarcinoma, were described as of very invasive and agressive phenotype, tumorigenic in athymic nude mice and able to metastasize in the lung (13). In contrast, MCF-7 is a hormone-dependent cell line, non-tumorigenic in nude mice and non-invasive in Matrigel (13). The effects of these cells on the HUVEC endothelium and on three major ECM components (secreted cell fibronectin, laminin and collagen IV) involved in cell adhesion and migration were also investigated.

Materials and Methods

Reagents and antibodies. All the culture media and additives were purchased from Invitrogen Corp. (Cergy Pontoise, France). Gelatin (type A porcine skin) was from Sigma (Saint Quentin Fallavier, France); the MMP inhibitor GM6001 was from Calbiochem (Merck, Nottingham, UK); polyacrylamide and all reagents used for electrophoresis were from Biorad S.A. (Ivry sur Seine, France) except the Novex Tricine gels (Molecular Probes Invitrogen Corp., Cergy Pontoise France); chemiluminescence detection was performed using an electrochemiluminescence (ECL) kit from EG Healthcare Amersham Biosciences Europe (Saclay, France). Highly purified MMP-2 was from Oncogene Science (Siemens healthcare Diagnostic, Cambridge, MA, USA).

The following antibodies were used: monoclonal mouse anti-human pan-cytokeratins antibody Beckman (Roissy en France, France) polyclonal rabbit anti-human von Willebrand Factor (vWF), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) and HRP-conjugated rabbit anti-goat IgG antibodies Alexa-594-conjugated goat anti-rabbit IgG and Alexa-488-conjugated donkey anti-mouse IgG antibodies (Molecular Probes Invitrogen Corp); polyclonal goat anti-human collagen IV antibody (Chemicon, Millipore, Saint-Quentin en Yvelines, France); polyclonal rabbit anti-human laminin antibody (Institut Pasteur, Lyon, France); monoclonal anti-human cellular fibronectin (Sigma) HRP-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Europe, Newmarket, UK); HRP-conjugated goat anti-mouse IgM (Calbiochem, Merck, Nottingham, UK); monoclonal mouse anti-TIMP-2 (Ab-1) and anti-MT1-MMP (Ab-1) antibodies (Oncogene Science).

Cell culture. The HUVEC were freshly isolated as previously described (14) and grown in MCDB-131 culture medium supplemented with 20% fetal calf serum (FCS). For all the experiments, HUVEC pooled from three umbilical cord preparations were used at their first passage only. The MDA-MB-231 and MCF-7 human breast carcinoma cell lines (purchased from American Tissue Cell Collection, ATCC-LGC, Molsheim, France) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. All the media were supplemented with 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 2.5 μg/ml of fungizone. The absence of mycoplasms was routinely checked and preparation of the cell suspensions was performed using 0.53 mM EDTA solution.

Co-culture and tumor cell transendothelial migration assay. Transwell cell culture chambers (Corning-Costar, Shiphol-Rijk, the Netherlands) consisting of polycarbonate filters (8 μm pore size; 0.33 cm^2 area) were used for coculture assay with a HUVEC monolayer grown on their lower side and tumor cells plated on their upper side. This co-culture system mimics the *in vivo* orientation of endothelial and tumor cells during tumor cell intravasation and allows tumor cells to make close contacts with HUVEC basal pole.

The HUVEC were first plated and grown to confluence (48 hours) on the lower side of the Transwell filter precoated with human plasma fibronectin. The HUVEC monolayer and the tumor cells to be used were then cultured in serum-free DMEM/F12 medium supplemented with 0.2% bovine serum albumin (BSA) for 4 hours prior to the assay. At this time (T_0), the tumor cells were plated onto the upper side of the filter at 5×10^4 cells/filter in the same serum-free medium. After incubation for 3, 7 or 24 hours, the upper and lower side's conditioned media were collected for zymographic and Western blot studies and the tumor cells that had migrated across the filter to the lower side were identified by immunostaining and counted. In parallel, two controls were performed in the same conditions: firsly tumor cells in monoculture to test their spontaneous migration when cultured alone; and secondly HUVEC monocultures, without the addition of tumor cells in the upper compartment, to assess the endothelial cell monolayer integrity during the 24 hours experiment. All the culture conditions were performed in triplicate.

In some experiments, the MMP inhibitor GM6001 was added to the medium for 24 hours on both sides of the filter, immediately after tumor cell plating and controls received equivalent dilution with DMSO vehicle alone.

Immunostaining. Tumor and endothelial cells present on the lower side of the filter were respectively distinguished by a double staining of pan-cytokeratins and vWF directly performed on the filter. Briefly, the filters were fixed for 10 minutes with 4% paraformaldehyde (PFA) and non-migrating tumor cells were removed by wiping the filter upper side of the filter with cotton swabs. The cells were then permeabilized with ice-cold acetone:methanol (1:1) for 5 minutes and incubated with 2% BSA for 30 minutes. A cocktail (1:1) of polyclonal vWF and monoclonal pan-cytokeratins antibodies was added to the cells overnight at 4˚C. After 3 washes, the filters were incubated with a cocktail (1:1) of Alexa-594 (red stain) and 488 (green stain)-conjugated secondary antibodies for 45 minutes. For negative controls, isotype-matched immunoglobulin was used. The nuclei were counterstained with (DAPI). Finally, the filters were cut off from their support, mounted on glass slides with coverslips and observed under a fluorescence microscope (Leica Microsystèmes Rueil Malmaison, France). The HUVEC and MDA-MB-231 control monocultures were respectively stained for vWF or pan-cytokeratins under the same experimental conditions. The immunostained cells were counted with the aid of an image analysis program (Visiolab-Biocom, Les Ulis France,). On each filter, the total cell number and percentage of tumor cells and HUVEC were evaluated in 25 predetermined fields (at magnification ×40). Results are expressed as the mean value±SD of four separate experiments. Statistical comparisons were performed using the Mann-whitney *U*-test (p-value <0.05 was considered statistically significant).

The concomitant HUVEC migration toward the filter upper side was also assessed after 24 hours of co-culture. After the removal of cells present on the filter lower side, HUVEC and tumor cells present on the filter upper side were identified as described and counted.

Scanning electron microscopy. The HUVEC were grown to confluence on the filter as above. The cells were removed in using a 0.1 N NH_4OH solution and the filters were fixed with 85 mM phosphate buffer pH 7.2 containing 4% paraformaldehyde and 1% glutaraldehyde. The preparations were post-fixed in 0.1% osmium tetraoxide, dehydrated in graded ethanol baths, dried to critical point using hexamethyldisilizane, and coated by platine sputtering. They were examined with a Zeiss Gemini (Carl Zeiss, Le Pecq, France) scanning electron microscope.

HUVEC basement membrane degradation. The HUVEC matrix degradation after 24-hour tumor cell transendothelial migration was evaluated by measuring the level of collagen IV, laminin and cellular fibronectin present in the HUVEC matrix with three specific ELISAs directly performed on the lower side of the Transwell filters. Briefly, the tumor cell transendothelial migration was first carried out as described in transendothelial migration assay concomitantly with a HUVEC monoculture used as HUVEC matrix reference. After 24 hours of culture, the cells present on the filters were removed using a 0.1 N NH_4OH solution and the levels of the three proteins in the exposed matrix were immediately analyzed using previously described *in situ* ELISA (15). The results were expressed as mean absorbance value (Abs)±SD, in each culture condition run in triplicate (statistical comparisons: Mann-Whitney *U*-test).

Zymography. The conditioned media (after 24-hour culture) normalized to protein level were mixed with non-denaturing Laemmli sample buffer and were subjected to electrophoresis, without prior reduction and heating, using 7.5% polyacrylamide gels co-polymerized with gelatin (0.12%) and SDS (0.1%). The gels were then washed twice for 30 minutes with 2.5% Triton® X100 and twice with water. After 20 hours incubation (37˚C) in 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$ substrate buffer, the gels were stained with Coomassie Blue R250 and then destained. Gelatinolytic activities appeared as white bands within the stained gel the intensity of the bands was determined using the Image J software. (www.uhnres.utoronto.ca/facilities/wcif/download.php). Control gels with the same samples were incubated in substrate buffer containing 20 mM EDTA to confirm the metalloproteinase activity. Purified pro-MMP-2 and conditioned medium from phorbol-12-myristate 13 acetate (PMA)-stimulated HUVEC (100 ng/ml, 24 hours) were used as latent and active MMP-2 positive controls.

Western blot analysis. After reduction and heating, the same normalized conditioned media and their related cell lysates prepared as described elsewhere (16) were respectively run on a 10-20% Tricine gel for tissue inhibitor metalloproteinase-2 (TIMP-2) detection and on a 7.5% acrylamide gel for MT1-MMP. The proteins were then transferred to a nitrocellulose membrane. Blocking of the membrane and probing with specific antibody (respectively at 5 µg/ml and 10 µg/ml, overnight at room temperature) were performed in TBS (0.05M Tris pH 7.4, 0.1 M NaCl) containing 0.05% Tween-20 and 5% creamed dry milk. After 1 hour incubation with HRP-conjugated secondary antibody in the same buffer, the proteins were visualized using an ECL kit. The relative intensity of the labeled band was measured using Image J software.

Results

Tumor cell intravasation. When the MDA-MB-231 cells were grown alone, 37.3±5.9% of the plated cells spontaneously migrated across the filter after 24 hours of incubation whereas, in coculture with the HUVEC, 58.7±11.0% of these tumor cells migrated towards HUVEC endothelium during the same time. This accelerated migration rate was nearly statistically significant after 24 hours of coculture (p=0.05) whereas no difference was observed up to the seventh hour of coculture (p=0.8) (data not shown).

At T_0, the HUVEC formed a confluent cell monolayer spreading over the entire available filter surface. During the subsequent hours, the HUVEC monolayer, in the absence of MDA-MB-231 cells, displayed only few spontaneous cell losses (approximately 10% after 24 hours). In contrast, coculture with MDA-MB-231 cells induced visible HUVEC monolayer damage as early as after 3 hours of incubation and an extensive loss of the HUVEC was observed after longer incubation periods. Thus only 17.2±7.9% of the HUVEC present at T0 remained on the filter at 24 hours of coculture (data not shown).

By comparing MDA-MB-231 cells with a less invasive breast carcinoma cell line, MCF-7, opposite results were obtained under the same conditions. Approximately 1% of plated MCF-7 cells spontaneously transmigrated across the filter after 24 hours and the presence of HUVEC did not induce a significant increase of their transmigration rate. No severe alteration of the HUVEC monolayer was observed in

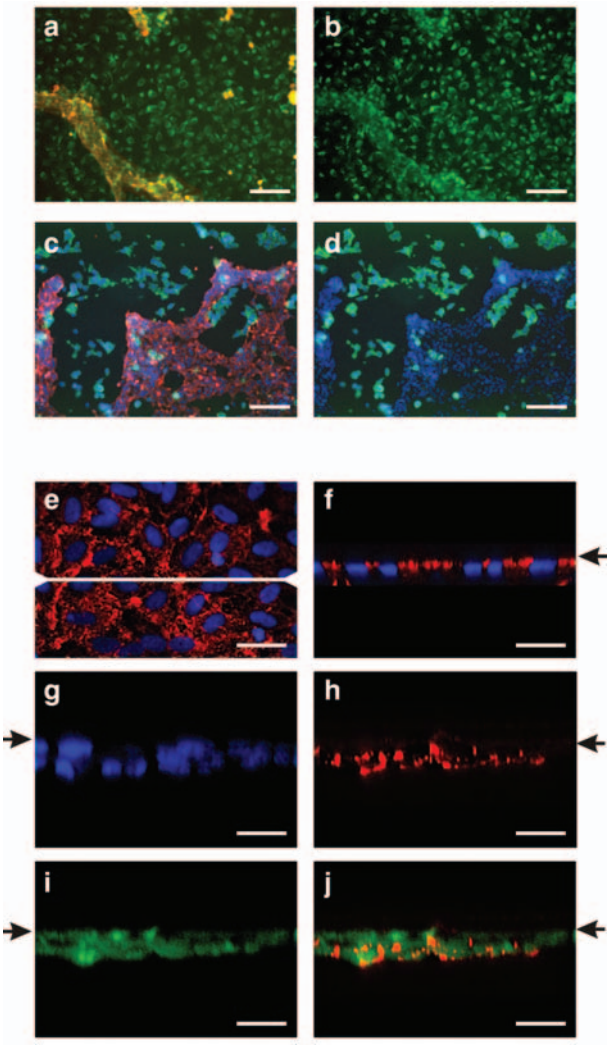

Figure 1. *Comparative MDA-MB-231 versus MCF-7 cell behaviour during their transendothelial migration. After 24 hours co-culture, Transwell filter lower sides were examined by fluorescence (a-d) and confocal (e-j) microscopy. a: Transmigrated MDA-MB-231 cells (green pan-cytokeratins) present even under HUVEC islets (red vWF) as assessed by the orange shade; b: corresponding field under FITC illumination; c: transmigrated MCF-7 cells (green) located outside the HUVEC sheets which exhibited a clear red colour; d: corresponding field under FITC illumination with nuclear DAPI staining aiding HUVEC location; Bar: 100 μm. HUVEC stained with vWF (red) and DAPI (blue) in e: control monoculture lateral view showing tightly cohesive endothelial cells and f: axial view (taken along the grey bar marked in e) of the same cells showing cell adhesion to the Transwell filter; g: DAPI nuclear staining (blue) and h-i: axial views of HUVEC-(red)-MDA-MB-231 cells (green) co-culture sequentially acquired under different laser wavelength illuminations and j: subsequently merged. Arrows indicate the Transwell filter. Bar:50 μm.*

the presence of MCF-7 cells since 78.2±13.1% of HUVEC remained on the filter after 24 hours of coculture (data not shown).

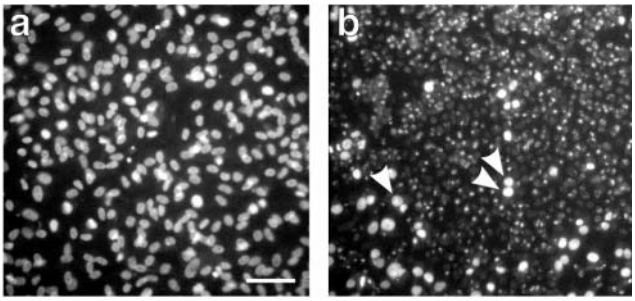

Figure 2. *HUVEC apoptotic nuclei assessed by DAPI cytochemistry a: HUVEC monoculture at 24 hours as control. b: HUVEC co-cultured with MDA-MB-231 cells. Fragmented apoptotic nuclei were widely observed only in vWF-positive HUVEC cells (arrowheads). The location of MDA-MB-231 nuclei is indicated (*). Bar: 50 μm.*

HUVEC anoikis. The lower sides of the double immunostained filters were observed under both optical (Figure 1a-d) and confocal (Figure 1e-j) microscopy. As seen in Figure 1a and b, transmigrated MDA-MB-231 cells (green staining) were found in space free of HUVEC (red staining) as well as under the endothelial islets. The orange colour resulted from the superimposition of green (pan-cytokeratins) and red (vWF) staining. In contrast, the rare transmigrated MCF-7 cells exhibited a completely different distribution pattern as they were observed beside the remaining HUVEC islets (Figure 1c, d). A confocal microscopy analysis was performed to investigate the HUVEC structure and their interaction with the MDA-MB-231 cells. When cultured alone, the HUVEC grew as a tight contiguous and regular cell monolayer with typical vWF staining (Figure 1e). The cross section of this monolayer (Figure 1f) showed that the HUVEC also exhibited tight contacts with the Transwell filter. After 24 hours co-culture, a cross section of the remaining HUVEC islets showed that the HUVEC no longer adhered to the filter (Figure 1h) and that transmigrated MDA-MB-231 cells an under the HUVEC monolayer (Figure 1i). A merged image of Figures 1h and i (Figure 1j) showed that the MDA-MB-231 cells were able to displace the HUVEC during their transendothelial migration.

The hallmarks of HUVEC apoptosis were then investigated throughout the cultures. In the HUVEC monocultures, only few and sparse condensed or fragmented nuclei (3 to 6% of the total HUVEC) were observed after a 24 hours of culture (Figure 2a). As early as 3 hours of co-culture with MDA-MB-231 cells, the HUVEC monolayer displayed small disseminated apoptotic islets (2.4±0.8% HUVEC exhibiting apoptotic nuclei) which increased in both number and size concomitantly with the formation of holes in the endothelial monolayer (as shown in Figure 1a). After 16 hours of co-culture, the HUVEC endothelium consisted

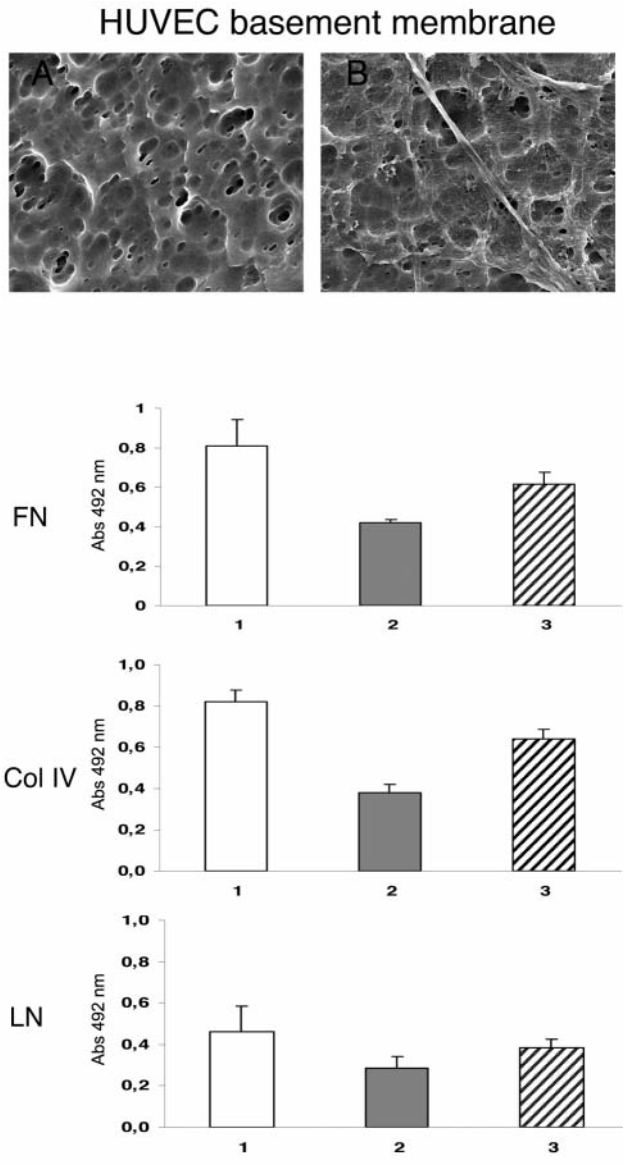

Figure 3. *Matrix laminin and collagen IV degradation in HUVEC matrix during MDA-MB-231 cell transmigration. Presence of a HUVEC basement membrane on the filters observed by scanning electron microscopy in the absence of cells (a) or after lysis of the HUVEC by ammoniac (b): in this case, fibrilla material is present on the surface of the filter. Fibronectin (FN), collagen IV (Coll IV) and laminin (LN) levels formed a continous network fluorescence as stained by their specific antibodies, in co-culture with MDA-MB-231 cells, the network was disrupted. Proteins assessed by ELISAs in matrices of HUVEC in monoculture (open bars) and in co-culture (striped bars) for 24 or 48 hours and in the presence of GM6001 (25 μM) (grey bars). The absorbance (Abs.) was measured at 492 nm. These data are representative of two independent experiments performed in triplicate (Student's t-test; *p=0.05).*

of about 88% of apoptotic endothelial cells (Figure 2b) whose detachment generated the wide HUVEC-free spaces observed later. This apoptosis was confirmed by electronic microscopic analysis of the HUVEC (data not shown). In contrast, co-culture with MCF-7 cells did not induce such cell detachment and only approximately 20% of the HUVEC nuclei exhibited the hallmarks of apoptosis after 24 hours of co-culture (data not shown).

A possible contribution of MDA-MB-231 cell soluble factor(s) to the HUVEC detachment and apoptosis was tested. Tumor cell conditioned medium instead of tumor cells was added for 24 hours into the upper compartment of the Transwell system. Neither MDA-MB-231 nor MCF-7 cell conditioned medium induced HUVEC detachment and/or apoptosis (data not shown). These results demonstrated the involvement of tumor-endothelial cell contact in the endothelial cell death events occurring in the tumor intravasation model.

HUVEC basement membrane degradation and MMP-2 activation. To confirm that an anoikis process was mainly involved in this HUVEC damage, both the HUVEC matrix integrity and the MMP-2 activities were investigated after 24 hour MDA-MB-231 cell transendothelial migration.

The HUVEC grown on the Transwell filters synthesized a basement membrane as shown by the presence of fibrillar material on the filter (Figure 3a). This fibrillar material composed of collagen IV, laminin and fibronectin was digested after 24 hours of co-culture as shown by the disrupted network stained by the different antibodies (Figure 3). ELISA assays revealed a significant loss in collagen IV, laminin and cellular fibronectin levels in the HUVEC matrix (p=0.05) after 24 hours of co-culture. Prolonged co-culture, up to 48 hours, confirmed the marked loss of these proteins. The plasma fibronectin level remained unchanged throughout this time (data not shown).

The activation of MMP-2 was investigated by zymographic analysis of the associated conditioned media. As shown in Figure 4a, HUVEC stimulated with PMA (positive control, lane 2) exhibited 2 lytic bands corresponding to the latent and active forms of MMP-2, whereas conditioned medium from MDA-MB-231 cells alone (lane 3) did not contain any MMP-2 activity. When cultured alone on the filter (lane 4), HUVEC showed a large lytic band corresponding to pro MMP-2 with a slight band of lower molecular weight identified as active MMP-2. This latter one was significantly increased (about 2-fold as assessed by densitometric analysis of more than 5 assays) in the presence of MDA-MB-231 cells (lane 5). All these gelatinolytic activities were blocked when EDTA was added to the incubation buffer, confirming the metalloproteinase activity (data not shown). Concomitant TIMP-2 and MT1-MMP levels were assessed by Western blot analysis respectively in the same conditioned media (Figure 4b) and in the lysates of the associated cells present on the lower side of the filters (Figure 4c). In HUVEC monoculture, TIMP-2 was secreted at a low constitutive

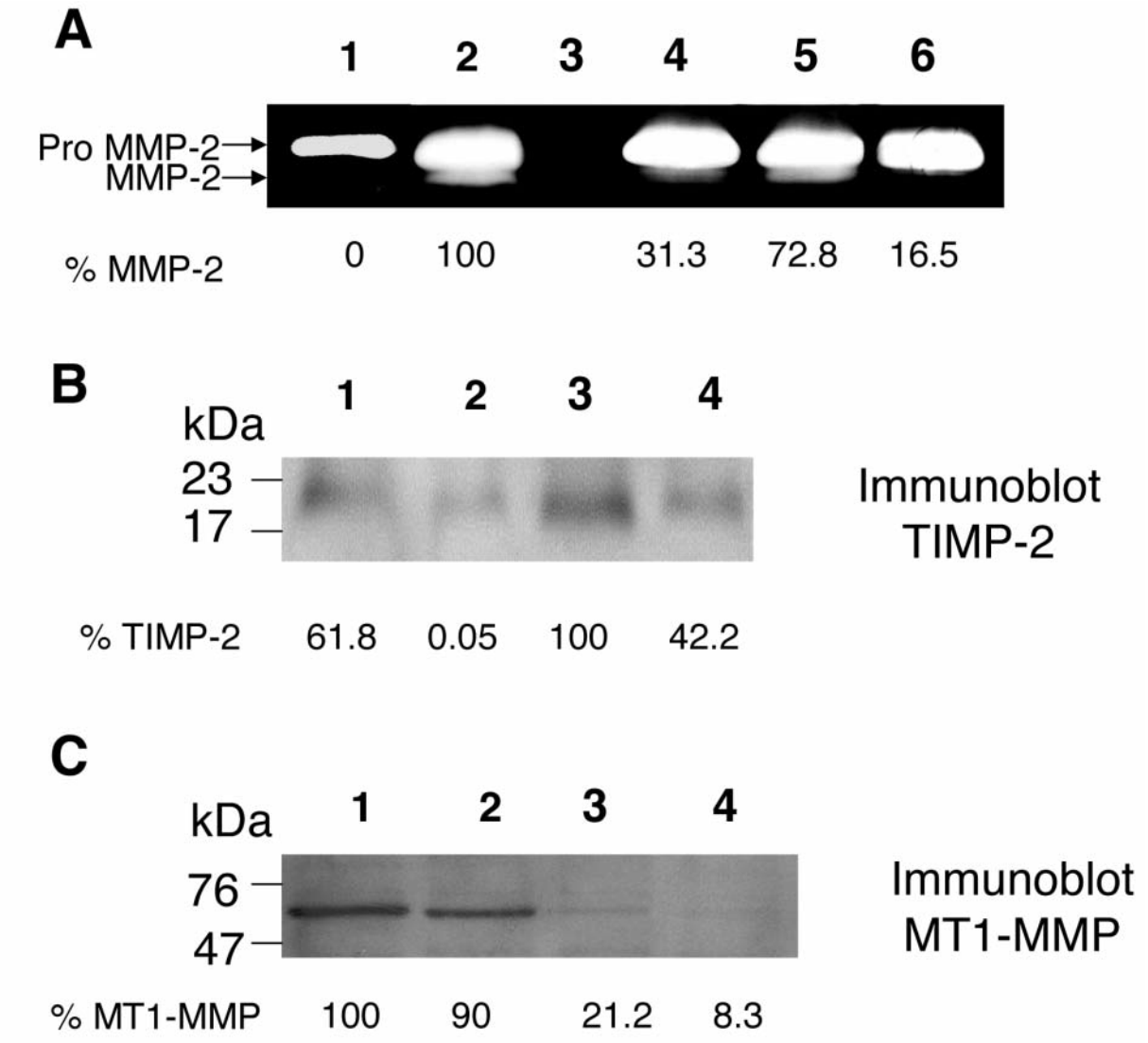

Figure 4. *MMP-2, TIMP-2, MT1-MMP expressions. a, Zymographic analysis of MMP-2 expression in conditioned media after 24-hour culture. Arrowheads indicate the latent and the active forms of MMP-2. Lane 1: highly purified pro-MMP-2; lane 2: PMA-treated HUVEC; lane 3: MDA-MB-231 cells alone; lane 4: HUVEC alone; lane 5: HUVEC/MDA-MB-231 cells coculture; lane 6: HUVEC/MDA-MB-231 cells co-cultured in the presence of GM6001. Western blot analysis of (b) TIMP-2 expression of the same conditioned media and (c) MT1-MMP expression of the lysates (10 μg proteins) of the associated cells present on the underside of the filter. b, Lane 1: HUVEC alone; lane 2: HUVEC/MDA-MB-231 cell co-culture; lane 3: HUVEC/MDA-MB-231 cells co-cultured in the presence of GM6001 (25 μM). c, Lane 1: HUVEC alone; lanes 2 and 3: MDA-MB-231 cells alone, present on the same filter respectively on the upper side (not yet migrated cells) and on the lower side (migrated cells); lane 4: HUVEC/MDA-MB-231 cells in co-culture. The relative percent age yield of MMP-2, TIMP-2 and MT1-MMP was determined by the percentage of pixels in each band comparing to the most intensely labeled band in each experiment.*

level (Figure 4b, lane 1). In co-culture, conditioned medium contained 2-fold enhanced TIMP-2 level (lane 2) and this increase was concomitant with the MMP-2 activation seen in Figure 4a (lane 5). In monocultures, HUVEC expressed high levels of MT1-MMP as did the MDA-MB-231 cells present on the upper side of the filter which had not yet migrated (Figure 4c, lanes 1 and 2). Conversely, the MDA-MB-231 cells which had migrated for 24 hours on the filter lower side, contained 2-fold less MT1-MMP (lane 3). In co-culture, the MTI-MMP level in the mixed cell lysate was nearly undetectable (lane 4).

The same experiments were also performed in the presence of GM6001 (25 μM), a broad-spectrum MMP inhibitor, added in the culture medium throughout the co-

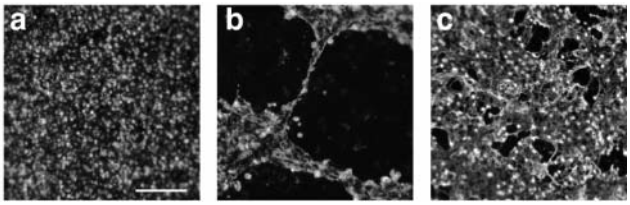

Figure 5. *Effect of GM6001 on HUVEC-MDA-MB-231 cells. HUVEC were grown in assay medium either alone (a) or in co-culture with MDA-MB-231 cells in the absence (b) or in the presence of GM6001 (c) for 24 hours. HUVEC were identified by vWF staining. Bar: 50 μm.*

culture assay. After 24 hours culture, the presence of GM6001 (i) prevented MDA-MB-231 cell intravasation and HUVEC monolayer damage, as only a few holes were observed in the HUVEC monolayer which remained adhered to the filter up to 24 hours (Figure 5); (ii) in the presence of MDA-MB-231 cells, the HUVEC matrix degradation as measured by levels of collagen IV and laminin remained to a significant extent ($p>0.05$) near to that of the matrix control (Figure 3); and (iii) the MMP-2 activation induced in the presence of MDA-MB-231 cells (Figure 4a, lane 6) was concomitant with a decrease in TIMP-2 secretion (Figure 4b, lane 3). These results demonstrated the great involvement of MMPs including the MMP-2-MT1-MMP complex, during the tumor cell intravasation and its concomitant endothelium disruption.

Discussion

It is currently admitted that tumor cell invasiveness depends mainly on the tumor cell phenotype. In the present study the very invasive breast tumor cells (MDA-MB-231) were attracted by the HUVEC endothelium within 24 hours of coculture, whereas under the same conditions, the non-invasive phenotype MCF-7 cells only weakly migrated through the HUVEC endothelium. These results were consistent with a previous report (17) showing that tumor cells were strongly attracted by HUVEC in a two- and three-dimensional chemotaxis systems. They differ from those of Lewalle *et al*. (18) who showed that HUVEC stimulated non-invasive MCF-7 cell transmigration in extravasation assays.

The present results also demonstrated that during tumor cell intravasation, HUVEC basement membrane may modulate the ability of MDA-MB-231 cells to migrate. In our model, when the MDA-MB-231 cells invaded the HUVEC monolayer, they lay under the HUVEC and, consequently, induced the detachment of the latter and their rapid apoptosis. This observation could be made by optical microscopy by 3 hours of co-culture and was confirmed by electron microscopy (data not shown). This process, termed anoikis, can be induced by disruption of the interactions between endothelial cells and the secreted ECM (19) and an important degradation of fibronectin, laminin and collagen IV, which are efficient substrates for cell adhesion and migration (20), was observed in this study.

ECM degradation has been shown to be affected by MMPs, particularly MMP-2 which can be activated by membrane type MMPs such as MT1-MMP in a ternary complex with TIMP-2 involved in cell migration and tumor dissemination (10). Since it was previously shown that MT1-MMP is activated when breast tumor cells migrate on laminin (12), MMP-2 activated by MT1-MMP or MT1-MMP itself could be responsible for the laminin degradation of the HUVEC basement membranes. Indeed in the present study, concomitantly with the transendothelisation of the MDA-MB231 cells and the HUVEC anoikis, the pro-MMP-2 was activated as early as after 24 hours of co-culture. The mechanism of this activation through the MMP-2/TIMP-2/MT1-MMP complex and how balance between deficiency and excess of TIMP-2 may regulate this activation and tumor cell invasion has been demonstrated (21). Under the present assay conditions, the HUVEC and MDA-MB-231 cells alone respectively secreted low level or no detectable TIMP-2 confirming previous report (22). In the MDA-MB-231 cell-HUVEC cocultures, TIMP2 which is also involved in pro-MMP-2 activation (21) was strongly enhanced, up to a level allowing the endothelial pro-MMP-2 activation. MT1-MMP expression in both cell types was also modified during the MDA-MB-231 cell intravasation. In the HUVEC as in the MDA-MB-231 cell monocultures, MT1-MMP was very abundant as reported before (10). However, we showed for the first time that only the MDA-MB-231 cells that had not yet migrated highly expressed MT1-MMP, whereas those present on the lower side of the Transwell filter after migration poorly expressed it, indicating that the MT1-MMP level was associated with the "migrating phenotype" of MDA-MB-231 cells. Thus the MT1-MMP-TIMP2-pro-MMP-2 complex on migrating cells results in MMP-2 activation and consequently ECM protein degradation by this MMP as well as cell migration and tumor dissemination (10). MT1-MMP overexpression at the tumor cell surface, which induces a marked increase in cell migration has been localized predominantly to invadopodia, specialized membrane extensions that are the sites of ECM degradation (23). Once MT1-MMP has activated pro-MMPs it is internalized by dynamin-endocytosis and degradated (24). These results may explain why the MDA-MB-231 cells did not contain any MT1-MMP in the membrane fraction after transendothelial migration. The fact that a pan-MMP inhibitor (GM6001) strongly reduced the MDA-MB231 transendothelial migration, HUVEC anoikis and laminin degradation confirmed a direct correlation between these three events.

Previous reports have shown a lack of both MMP-2 and MT1-MMP expression in MCF-7 cells (25, 26), though low MMP-2 expression has sometimes been mentioned, together with these cells's incapacity to activate exogenous MMP-2 (27). On the other, Lewalle *et al*. (28) demonstrated MCF-7 cell ability to disrupt endothelial cell intercellular junctions in an extravasation model. These results could explain why, in our intravasation model, the MCF-7 are poorly invasive and the migrated cells were always observed beside remaining HUVEC islets and never below them.

In our intravasation model, as observed by others in extravasation models (8), endothelial apoptosis required endothelial-tumor cell contacts since conditioned MDA-MB-231 medium cannot reproduce this phenomenon. Moreover, MDA-MB-231-HUVEC cell communications were visualized by dye exchanges, probably occurring by gap junctions (data not shown). Thus, cytoplasmic exchanges between tumor cells and HUVEC could be in part responsible for this apoptosis though the possibility that factors such as PAF and other cytokines, produced by certain tumor cells, which could be involved in the tumor-endothelial cell interactions, apoptosis and neoangiogenesis cannot be definitely excluded.

In conclusion, agressive and invasive MDA-MB-231 breast tumor cells can intravasate the endothelium by activating the MT1-MMP-MMP-2-TIMP2 complex, which degrades laminin, collagen IV and cellular fibronectin and subsequently detaches the endothelial cells, thus inducing endothelium apopto-necrosis. Thus, in this model mimicking the intravasation process through the basal membrane of endothelial cells, proliferating tumor cells can induce the necroapoptosis of endothelial cells observed during tumor progression.

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