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Induction of Endothelial Cell Apoptosis by Solid Tumor Cells

Florence Kebers,* Jean-Marc Lewalle,* Joëlle Desreux,* Carine Munaut,* Laetitia Devy,†
Jean-Michel Foidart,* and Agnès Noel*

** Laboratory of Biology, University of Liège, B-4000 Liège, Belgium; and †Laboratory of Biochemistry, University of Reims, F-51096 Reims, France*

Abstract: The mechanisms by which tumor cells extravasate to form metastasis remain controversial. Previous studies performed *in vivo* and *in vitro* demonstrate that the contact between tumor cells and the vascular wall impairs endothelium integrity. Here, we investigated the effect of breast adenocarcinoma MCF-7 cells on the apoptosis of human umbilical vein endothelial cells (HUVEC). TUNEL labeling, nuclear morphology, and DNA electrophoresis indicated that MCF-7 cells induced a two- to fourfold increase in HUVEC apoptosis. Caspase-3 activity was significantly enhanced. Neither normal cells tested (mammary epithelial cells, fibroblasts, leukocytes) nor transformed hematopoietic cells tested (HL60, Jurkat) induced HUVEC apoptosis. On the contrary, cells derived from solid tumors (breast adenocarcinoma, MDA-MB-231 and T47D; fibrosarcoma, HT 1080) had an effect similar to that of MCF-7 cells. The induction of apoptosis requires cell-to-cell contact, since it could not be reproduced by media conditioned by MCF-7 cells cultured alone or cocultured with HUVEC. Our results suggest that cells derived from solid tumors may alter the endothelium integrity by inducing endothelial cell apoptosis. On the contrary, normal or malignant leukocytes appear to extravasate by distinct mechanisms and do not damage the endothelium. Our data may lead to a better understanding of the steps involved in tumor cell extravasation.

INTRODUCTION

Hematogenous metastasis is a complex process including (1) penetration of malignant cells into blood circulation, (2) tumor cell adhesion to endothelial cells, (3) tumor cell migration through the endothelial cell lining and the basement membrane, and (4) invasion of surrounding tissues [1]. The time sequences and mechanism by which tumor cells cross endothelial and subendothelial barriers to reach the extravascular space (extravasation) remain controversial. Individual tumor cells may extravasate with only little damage to microvasculature in a manner similar to leukocytes [2]. In this context, *in vivo* videomicroscopy observations of mouse liver and chick chorioallantoic membrane suggest that cancer cells extravasate singly without observable disruption of the microvasculature [3, 4]. Alternatively, studies performed *in vivo* have shown that tumor cells interacting with platelets adhered to endothelium proliferated intravascularly and penetrated into surrounding tissues by degrading the basement membrane [5, 6].

In vivo, morphological observations of early lung metastases have evidenced degenerating endothelial cells adjacent to the attached tumor cells [5]. Accordingly, *in vitro,* activated pancreatic tumor cells have been shown to damage endothelial cells [7]. We and others have previously shown that tumor cells adhere to the endothelial cell monolayer and induce endothelial cell retraction [8-11]. This process was accompanied by transient modifications in the intracellular free Ca^{2+} concentration in endothelial cells [12]. Furthermore, modifications of adherens junction protein distribution and tyrosine phosphorylation were observed and correlated with loss of interendothelial cohesion and endothelial cell detachment [13]. Taken together, these *in vitro* and *in vivo* observations suggest that some tumor cells may impair endothelium integrity.

Apoptosis is a highly ordered cell death process characterized by nuclear alterations (chromatin condensation, internucleosomal DNA cleavage), ultrastructural modifications (cytoskeletal disruption, cell shrinkage, and membrane blebbing), and biochemical changes, such as activation of members of the interleukine-1-β converting enzyme protease family recently named "Caspases" [14]. The whole process leads to fragmentation of dying cells into apoptotic bodies that are phago-cytosed by neighboring cells or macrophages [15-17].

The present study was designed to investigate the potency of normal and different tumor cell types to affect endothelial cells viability. Apoptosis was investigated by TUNEL labeling, nuclear staining, DNA electrophoresis, and activity quantification of Caspase 3 (CPP32/apopain/YAMA).

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MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated by umbilical vein treatment with 0.1% collagenase (from *Clostridium histolyticum;* Sigma, St. Louis, MO) for 15 min at 37°C [18]. HUVEC aggregates were plated on gelatin-coated dishes and cultured in RPMI 1640 (GIBCO) supplemented with 5% heat-inactivated fetal calf serum (FCS) (GIBCO), 5% heat-inactivated pooled human serum, 20 μg/ml endothelial cell growth factor from bovine brain, (Boehringer Mannheim), 50 μg/ml heparin (Sigma), 10 mM Hepes, and penicillin-streptomycin 100 IU/ml (Merck). Cells reacted positively with an antibody to Factor VIII-related antigen and showed the typical cobblestone morphology when confluent. To avoid phenotypic drift due to prolonged time in culture, HUVEC were always used at the first passage.

Human mammary tumor cell lines MCF-7 and MDA-MB-231 were provided by G. Leclercq (Institut Bordet, Brussels, Belgium) and M-F. Poupon (Centre de Recherche sur le Cancer, Villejuif, Paris, France), respectively. These cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 5% heat-inactivated FCS, glutamine (292 mg/L) (Merck), sodium bicarbonate (2.1 mg/L) (Merck), ascorbic acid (50 mg/L) (GIBCO) and penicillin-streptomycin (100 IU/ml) (GIBCO). Medium was changed every 3 or 4 days and cells were passaged every week. For coculture experiments subconfluent tumor cell cultures were used.

Human promyelocytic leukemia HL60 cells were kindly provided by P. Jeannesson (Laboratory of Biochemistry, University of Reims, France) and were maintained in DMEM containing 15% FCS and penicillin-streptomycin (100 IU/ml).

Human T lymphocyte Jurkat cells were obtained from the American Tissue Cell Collection and were grown in RPMI (GIBCO) supplemented with 10% FCS and penicillin-streptomycin (100 IU/ml).

Normal mammary epithelial cells were obtained from reduction mammoplasty and processed immediately after surgery as previously described [19]. Primary cultures were performed in Joklik-modified Eagle's medium supplemented with 10% heat-inactivated FCS, glutamine (2 mM) (GIBCO), nonessential amino acids (10 ml/L) (GIBCO), insulin (10 μg/ml) (GIBCO), cholera toxin (100 ng/ml) (Sigma), Cortisol (0.5 μM) (Sigma), epidermal growth factor (20 ng/ ml) (Sigma), and 100 IU/ml penicillin-streptomycin (GIBCO). Culture medium was changed every 3 days and the primary cells used in the experiment were collected by trypsin treatment of a 15-day -old culture.

Normal human fibroblasts were provided by C. Lambert (Laboratoire de Biologie des Tissus Conjonctifs, Liège, Belgium). They were obtained by outgrowth from human skin explants as previously described [20] and used between passages 4 and 14.

Human leukocytes were isolated from peripheral blood from volunteer donors using Lymphoprep tubes (Nycomed) according to the manufacturer's instructions [21]. They were immediately used for experiments.

Cell attachment to HUVEC monolayers

HUVECs harvested from monolayers were seeded on 0.1% gelatin-coated separable wells of 8-well strips (40,000 cells/surface) and maintained in assay medium for 16 h to allow formation of a confluent monolayer. Cells to be tested were radiolabeled with ^{111}In, essentially as previously reported [22]. Briefly, cells (10^6 cells/ml) were incubated for 15 min at room temperature with 10 μCi of ^{111}In tropolonate in DMEM supplemented with 10% FCS, washed three times, and resuspended in assay medium. HUVEC were rinsed once with assay medium and overlaid with 100 μl of ^{111}In-radiolabeled cells (10^4 cells) in assay medium. The plates were incubated at 37°C in a 5% CO_2 atmosphere for various periods of time. Nonattached cells were removed by three washings with RPMI medium. The wells were separated, and the radioactivity of the adherent cells in each individual well was counted in a gamma counter (Beckman, San Mamon, CA).

Coculture experiments.

HUVEC (5 x 10^5 cells/dish) were plated onto gelatin-coated 35-mm-diameter dishes (Nunc) and grown to confluence in "HUVEC medium." Cells to be tested (MCF-7 tumor cells or other cells) were shortly trypsinized, washed once in DMEM supplemented with 5% FCS, and allowed to recover in RPMI 1640 medium supplemented with 5% heat-inactivated FCS and 5% heat-inactivated human serum ("assay medium") at 37°C

for 30 min. Cells (4 x 10^5) were added to endothelial monolayers in assay medium and incubated at 37°C for 20 h. Control endothelial monolayers were cultured alone, under the same conditions as in assay medium. Control MCF-7 cell (4 x 10^5) monocultures were also plated onto gelatin-coated dishes.

For quantification of cell detachment, cells were collected by centrifugation of the culture medium and counted after 6, 10, 20, 30, and 36 h of coculture. The endothelial origin of detached cells was assessed by Factor VIII-related antigen labeling as described below.

For serum-deprived conditions, MCF-7 cells and endothelial monolayers were extensively washed with preheated RPMI 1640 supplemented with 0.1% bovine serum albumin (BSA, GIBCO) before the plating of MCF-7 cells on HUVEC monolayers and incubation at 37°C for 15 h.

For cocultures with normal leukocytes, some HUVEC monolayers and control monolayers were pretreated overnight with 20 IU/ml interleukin-1 β (human recombinant IL-1β; Genzyme) [23].

In some assays, normal epithelial mammary cells or fibroblasts were grown to confluence in order to obtain monolayers. After addition of MCF-7 cells to these monolayers, cocultures were incubated for 20 h at 37°C.

TUNEL labeling and immunofluorescence

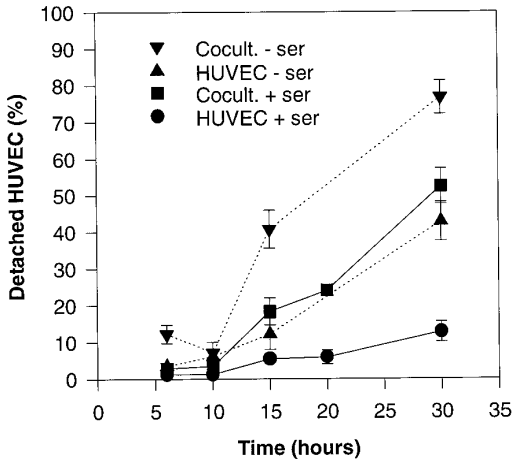
TUNEL was used to detect free 3'OH groups *in situ* in fragmented DNA according to the manufacturer's instructions *(in situ* cell death detection kit, Boehringer Mannheim) with some modifications: cocultures and monocultures were fixed in paraformaldehyde 4% in phosphate-buffered saline (PBS) at room temperature for 15 min and permeabilized with ice-cold methanol at -20°C for 10 min. Cells were rinsed with ethanol at 70°C and rehydrated twice with PBS for 10 min. Thereafter, cells were incubated with TUNEL reaction mixture (containing terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides) for 1 h. Preparations were rinsed and exposed to rabbit anti-human Factor VIII-related antigen antibody (Dako) (dilution 1:100 in 1% PBS-BSA) for 1 h. Rhodamine-conjugated murine anti-rabbit immunoglobulins (Dako) were used as secondary antibody at a dilution 1:30 in BSA 1% for 30 min. Nuclear counterstaining was finally performed with propidium iodide (50 ng/ml in PBS) for 30 min. Labeled cells were mounted in 50% glycerol and observed with a VANOX fluorescence microscope (Olympus).

The number of apoptotic HUVEC (both TUNEL and factor VIII-related antigen positive) or MCF-7 cells (factor VIII-related antigen negative) was counted as well as the total number of HUVEC and MCF-7 cells for each field (magnification, 100x). At least 1500 cells were counted for each preparation.

CPP-32 activity

CPP-32 activity was measured as previously described [24]. HUVEC were plated onto 85-mm-diameter gelatin-coated dishes and grown to confluence (3 X 10^6 cells). Monolayers were cocultivated with 2.4 X 10^6 MCF-7 cells in assay medium for 20 h as described under Coculture Experiments. The control HUVEC monolayer and 2.4 X 10^6 control MCF-7 cells were separately cultured under the same conditions. For lysis, cells were briefly rinsed in PBS, scraped, and placed on ice for 2 h in lysis buffer containing protease inhibitors (50 mM Tris, pH 7.4, 1 mM EDTA, 10 mM EGTA, 0.2 mM PMSF). Samples were centrifuged at 1500*g* for 10 min, and then supernatants were kept on ice. Protein concentration was brought to 90 μg/ml in AFC-120 buffer (50 mM Hepes, pH 7.5, 1% sucrose, 0.1% Chaps). Dithiothreitol was added to a final concentration of 10 mM. CPP-32 fluorogenic substrate Z-DEVD-AFC (AFC-138; Enzyme Systems Products) was diluted in DMSO (2.5 mM) and added to samples (final concentration 50 μM). Fluorescence changes were monitored with a Perkin-Elmer MPF-44B fluorescence spectrophotometer (excitation wavelength, 400 nm; emission wavelength, 505 nm) for 24 h. Lysates of freshly isolated or overnight cultured murine thymocytes were used as negative and positive controls, respectively [25].

Fig.1: *HUVEC detachment as a function of time. HUVEC were cultured alone (HUVEC) or with MCF-7 cells (Cocult.) in the presence (solid lines) or in the absence (dotted lines) of serum. Error bars indicate standard deviation for triplicate cultures.*



Preparation of conditioned media.

MCF-7 cells were grown to subconfluence in 75-cm^2 flasks (Nunc). They were washed twice in PBS and incubated with 10 ml assay medium. After 24 h, conditioned medium was harvested, centrifuged to remove cell debris, and stored at 4°C.

Media conditioned by MCF-7-HUVEC coculture and control HUVEC monolayers were harvested after 20 h of incubation and handled as described above.

DNA preparation and agarose gel electrophoresis.

HUVEC were seeded in 85-mm-diameter gelatin-coated dishes and grown to confluence (3 X 10^6 cells). Cocultures with 2.4 X 10^6 MCF-7 cells per dish were performed in RPMI 1640 supplemented with 0.1% BSA as described under Coculture Experiments. Control HUVEC monolayers and control MCF-7 cells were separately cultured under the same conditions. After 15 h, dishes were rinsed twice with PBS and attached cells were scraped in cell lysis buffer (50 mM Tris, pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS). Cell extracts from separated control HUVEC and MCF-7 cells were pooled. DNA was isolated as previously described [25, 26]. Then 30 µg DNA of each sample (coculture or control pool) was loaded on a 1.8% agarose gel. As a positive control, DNA was extracted from thymocytes cultured overnight in RPMI medium supplemented with 10% FCS, which leads to 25% apoptotic cells [25]. To obtain a ladder reference, 15 µg DNA of this extract was loaded on an agarose gel.

RESULTS

MCF-7 Cells Induce HUVEC Apoptosis

When seeded on HUVEC confluent monolayer in 10% serum medium, human breast adenocarcinoma MCF-7 cells adhered to endothelial cells and induced their retraction. This led to the formation of "holes" in the endothelial monolayer around the tumor cells [13]. MCF-7 cells spread on the exposed subendothelial matrix and induced HUVEC detachment. As illustrated in Fig. 1, 24 ± 1.5% of HUVEC had detached after 20 h, compared to 5.9 ± 1.9% in monocultures. After 30h, the percentage of HUVEC detachment in cocultures reached 52.5 ± 4.9%, while it was less than 13% in control monolayers (Fig. 1).

Apoptosis was analyzed by TUNEL labeling in MCF-7 cells or HUVEC monocultures, as well as in cocultures of both cell types (Fig. 2). When MCF-7 cells were cultured alone, less than 0.1% nuclei were TUNEL positive. In HUVEC monolayers, the number of TUNEL-positive cells ranged from 1.3 to 7.3% (Table 1). In the corresponding cocultures, there was a two- to fourfold enhancement of TUNEL-positive HUVEC (Figs. 2A, 2B, 2C, 3, and Table 1). TUNEL-positive cells displayed highly condensed and/or fragmented nuclei; this was further confirmed by counterstaining with propidium iodide (Figs. 2D and 2F). All TUNEL-labeled cells were Factor VIII-related antigen-positive, revealing their endothelial origin (Fig. 2E). In most cases, TUNEL-positive

HUVEC were in direct contact with MCF-7 cells (Figs. 2D and 2E). In some assays, cells detached after 20 h of coculture were collected. All of these cells were Factor VIII-related antigen and TUNEL positive (data not shown).

Further assays were performed in the absence of serum. Since these experimental conditions promoted the retraction and detachment of HUVEC induced by MCF-7 cells (Fig. 1), we reduced to 15 h the duration of cocultures. Under these conditions, the basal range of TUNEL-positive HUVEC cells was higher (5 to 12%), but a threefold increase of TUNEL-positive HUVEC was also recorded in cocultures (Table 1 and Fig. 3).

DNA was extracted from HUVEC and MCF-7 monocultures as well as HUVEC-MCF-7 cell cocultures and subjected to electrophoresis in a 1.8% agarose gel. In cocultures performed in the presence of serum, no DNA laddering was observed; this could be related to the small proportion of DNA from apoptotic HUVEC among intact HUVEC and MCF-7 cell DNA. However, in the absence of serum, which led to higher apoptotic cell numbers (Table 1), a typical laddering was seen in cocultures but not in controls (Fig. 4). DNA extracted from floating cells in cocultures also revealed an inter-nucleosomal fragments pattern (Fig. 4).

In order to further study the biochemical characteristics of the HUVEC apoptosis observed in the presence of MCF-7 cells, we quantified the Caspase-3 activity by using a specific fluorogenic substrate. In cellular extracts from cocultures, we found an increased activity, compared to separated monocultures of control HUVEC and MCF-7 cells pooled prior to extraction (Fig. 5).

HUVEC Apoptosis Is Not Related to the Secretion of a Soluble Factor by MCF-7 Cells

In order to determine whether a soluble factor(s) released by tumor cells was responsible for the induction of HUVEC apoptosis, we tested the effect of medium conditioned by MCF-7 cells on HUVEC monolayers. MCF-7 cell conditioned medium did not induce any retraction or HUVEC detachment and had no effect on HUVEC apoptosis. Since the secretion of a soluble apoptosis inducer may have been triggered by contacts between HUVEC and MCF-7 cells, conditioned medium was collected from MCF-7-HUVEC cocultures. It had no effect on HUVEC monolayers either (Fig. 3). Thus, these results suggest that cell-to-cell contacts play a key role in the apoptotic events reported here.

Table 1: *Percentage of Apoptotic HUVEC in MCF-7-HUVEC Coculture*

Assays	Control percentage of apoptotic HUVEC	Coculture percentage of apoptotic HUVEC	Coculture percentage of control
With serum			
1	1.3	5.1	392
2	2.8	7.1	255
3	2	3.9	193
4	7.3	23.4	322
5	2.3	8.6	374 Mean ± SD: 307 ± 83 (a)
Without serum			
1	5.4	16.3	302
2	11.9	32	269 Mean ± SD: 285 ± 23

Note. HUVEC were either cultured alone (Control) or cocultured (Coculture) for 20 h in the presence or in the absence of serum. Only factor VIII-related antigen positive and TUNEL-labeled cells with condensed and/or fragmented nuclei were counted as "apoptotic HUVEC." To allow comparison between separate experiments, the apoptotic fractions in endothelial control monolayers were set as 100% and the values after addition of MCF-7 cells were expressed as a percentage of the control (last column) a, $0.0001 < P$ 0.001 *(t* test).

Adenocarcinoma and Fibrosarcoma Cells, but Not Normal Mammary Cells or Fibroblasts, Induce HUVEC Apoptosis

Among other cellular types seeded on HUVEC monolayers (Table 2), the two other human mammary adenocarcinoma cell lines, MDA-MB-231 and T47D, induced HUVEC retraction and detachment and induced a two- to fourfold enhancement of apoptotic HUVEC number, similarly to MCF-7 cells (Table 2). Another cell line derived from solid tumors, the fibrosarcoma HT-1080 cell line, was also tested on HUVEC monolayers. The retraction and detachment of HUVEC appeared more rapidly than with adenocarcinoma cells. After 14 h of

coculture with HT-1080 cells, the extent of HUVEC retraction and apoptosis was similar to that observed after 20 h of coculture with MCF-7 cells. Normal human mammary epithelial cells, as well as normal fibroblasts, had no influence on HUVEC detachment or apoptosis (Table 2), even after a longer coculture time (48 h) (data not shown).

Normal and Transformed Hematopoietic Cells Do Not Induce HUVEC Apoptosis

Human leukocytes seeded on untreated or overnight IL-1β preactivated HUVEC monolayers did not induce any retraction, detachment, or apoptosis of endothelial cells (Table 2). The T-lymphoblastoid Jurkat cells and the human promyelocytic HL60 cells were also seeded on HUVEC monolayers. Similarly to normal leukocytes, these tumor cells did not induce HUVEC retraction or apoptosis.

MCF-7 Cells Do Not Induce Normal Mammary Epithelial Cell or Fibroblast Apoptosis

We further tested whether MCF-7 cells were able to induce the apoptosis of cell types other than HUVEC. When normal mammary epithelial cells or fibroblasts were used as targets for MCF-7 cells, no alteration of the monolayer integrity occurred. Furthermore, we did not observe any modification in the number of TUNEL-positive cells in these cocultures (data not shown).

Fig.2: *(A, B, and C) TUNEL labeling of MCF-7 cells and HUVEC maintained in monoculture or in coculture. HUVEC and MCF-7 cells were cocultured for 20 h as described under Materials and Methods (A). Monocultures of HUVEC (B) or MCF-7 cells (C) were used as controls. Apoptosis was visualized by TUNEL-FITC labeling (bright points). At higher magnification, each bright point corresponds to a condensed or fragmented nucleus (D and F). (D and E) TUNEL labeling and Factor VIII-related antigen staining of MCF-7 cells-HUVEC cocultures. HUVEC and MCF-7 cells (M) were cocultured for 20 h. Only HUVEC were labeled both by TUNEL method (D, yellow) and by polyclonal antibody raised against Factor VIII-related antigen (E, red) (arrows). (F) Propidium iodide staining of HUVEC-MCF-7 cells coculture showing the aspect of apoptotic nucleus (arrow). Bars correspond to 50 µm.*

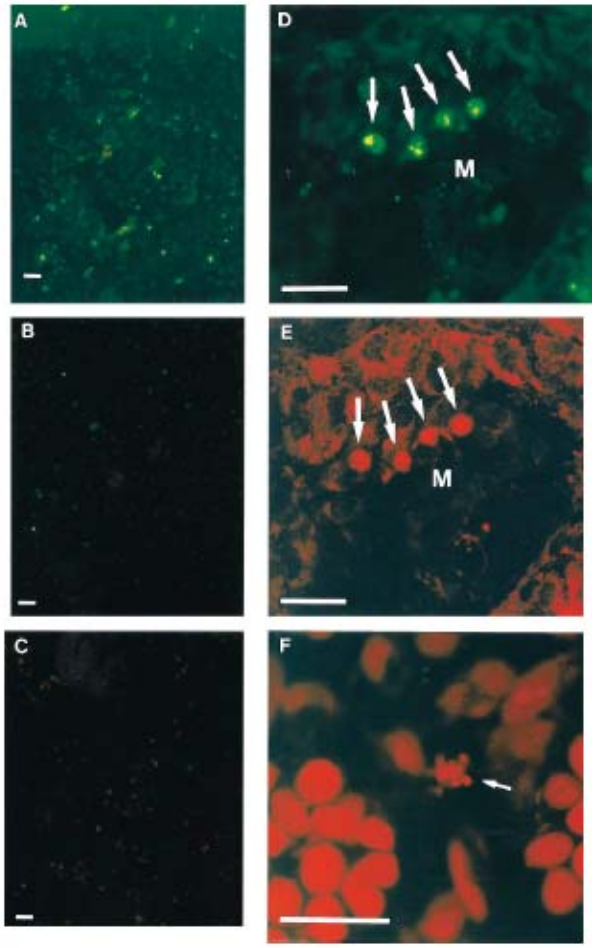
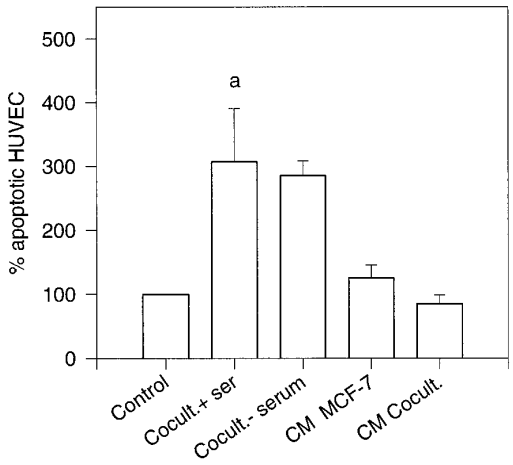


Fig.3: *Importance of cell-to-cell contacts in endothelial cell apoptosis induction. HUVEC were cultured in different conditions: alone (Control), in coculture with MCF-7 cells for 20 h with serum (Cocult. + serum), in coculture with MCF-7 cells for 15 h without serum (Cocult. — serum), and in the presence of medium conditioned by MCF-7 cells cultured alone (CM MCF-7) or by MCF-7 cells cocultured with HUVEC (CM cocult.). Apoptosis was quantified by TUNEL labeling as described under Materials and Methods. Apoptosis results were expressed in terms of percentage of corresponding control values, a, 0.0001 < P < 0.001 (t test).*



DISCUSSION

The interaction of tumor cells with vascular endothelium precedes their extravasation into target organs. The damage to the endothelium following tumor cell adhesion remains controversial. We have previously demonstrated that breast adenocarcinoma MCF-7 cells were able to adhere to endothelial cells and to induce their retraction [13]. The present study shows that endothelial retraction is associated with apoptosis of HUVEC. This apoptosis increase was observed in the presence or absence of serum and is additive to the higher basal rate of apoptosis recorded in serum deprivation conditions [27].

Apoptosis of endothelial cells was assessed by several classical criteria: generation of free 3'-hydroxyl ends in fragmented DNA (TUNEL labeling), nuclear condensation and fragmentation (propidium iodide), and DNA laddering on agarose gels, one of the most reliable biochemical markers of apoptosis [26]. The endothelial origin of TUNEL-positive apoptotic cells was assessed by Factor VIII-related antigen staining. Apoptotic MCF-7 cells were never observed.

Apoptotic cell death results from transduction of death signals triggered by various exogenous stimuli such as radiations, cytokines [i.e., tumor necrosis fac-tor-α (TNFα)], hormone withdrawal, growth factor or serum deprivation, loss of extracellular matrix or intercellular contacts, and Fas/Fas ligand signaling [17, 28-31]. In our model, since apoptosis appeared associated with cell retraction and detachment, we could hypothesize that cell death resulted from loss of adhesion to extracellular matrix. Indeed, it is well known that HUVEC and epithelial cells undergo apoptosis in the absence of interactions with extracellular matrix [27, 28]. While in our model, floating cells were apoptotic as assessed by TUNEL labeling and DNA laddering; we provided evidence that HUVEC which were still attached were also apoptotic (Figs. 3, 4, and 5 and data reported in Tables 1 and 2). Therefore, our data strongly suggest that the induction of apoptosis precedes HUVEC detachment.

The importance of cell-to-cell interactions was determined by using medium conditioned by MCF-7 cells or by MCF-7 cells cocultured with HUVEC. Since both conditioned media failed to affect HUVEC integrity and viability, apoptosis induction cannot be related to secretion of soluble factors by MCF-7 cells. These data emphasized the role of cell-to-cell interactions in these apoptotic events. This is further supported by the fact that apoptotic HUVEC were mostly observed in the vicinity of tumor cells. In developmentally programmed capillary regression, endothelial cell apoptosis dependent on contact with macrophages has recently been described [32]. T-cell-mediated cytotoxicity was shown to occur via perforin- and Fas-based mechanisms [29]. Fas ligand also appears to be expressed by hepatocellular carcinoma cells and by lung carcinoma cells, inducing apoptosis of T lymphocytes entering in contact with these tumor cells [33, 34]. In our model, it seems unlikely that Fas signaling mediates endothelial apoptosis since (1) preliminary experiments indicate that MCF-7 cells tested in the present study do not express Fas ligand (personal unpublished data), (2) HUVEC have been shown to express unfunctional Fas [35], and (3) in our experiments, MCF-7 cells did not induce apoptosis of normal

mammary epithelial cells, which express functional Fas [36]. Another mechanism possibly involved in our model could be the disruption of interendothelial contacts following interaction with tumor cells. This is supported by the fact that (1) in our model, tumor cells were able to alter interendothelial adherens junctions [13] and (2) colon tumor cells were shown to trigger an apoptotic death program after disruption of their intercellular contacts [30, 31].

Modification of the intracellular concentration of calcium is an intracellular event frequently involved in the apoptotic process [15, 37, 38]. In this context, we have recently shown that the contact between MCF-7 cells and HUVEC induced an immediate and transient increase in HUVEC intracellular free calcium concentration [12]. However, the potential link between intracellular calcium increase and apoptosis observed in our model remains to be ascertained.

Apoptosis is mediated by multiple pathways that involve a complex array of biochemical regulators and molecular interactions. Although the upstream signaling of apoptosis is uncertain, the family of cystein proteases related to *Caenorhabditis elegans* ced-3 gene, Caspases [14], seems to be play a key role in the apoptotic process. In particular, Caspase-3 (CPP32, apo-pain, YAMA) has been implicated in cell death induced by TNFα and Fas-mediated apoptosis [16, 39, 40]. By measuring the cleavage of a specific fluorogenic substrate [16], we found a significant increase of the Caspase-3 activity in coculture extracts, compared to the control extracts.

The involvement of a selective signaling pathway between tumor cells and HUVEC is supported by the fact that the viability of two other normal cellular types tested (mammary epithelial cells and fibroblasts) was not impaired by a 24-h coculture with MCF-7 cells. These data suggest the implication of specific interactions between MCF-7 cells and HUVEC.

Tumor cell interactions with endothelium have often been compared with leukocyte extravasation. The adhesion of leukocytes to endothelial cells has been described as a three-step event including tethering, rolling, and firm adhesion of leukocytes [41]. This model, first described for neutrophils, involved selection in the first two phases, while integrin (VLA_4) and their count-erreceptors of the IgG superfamily (ICAM-1 and V-CAM1) were thought to be implicated in the arrest phase. In contrast, it appeared more recently that VLA_4 can mediate both tethering and arrest of lymphocytes [42]. These observations strongly suggest that the mechanisms and the molecules involved in interactions with endothelial cells may differ from one cell type to another. Such a diversity of mechanisms is supported by our data demonstrating that adenocarcinoma cells and fibrosarcoma cells were able to induce HUVEC apoptosis, while normal or tumoral hematopoietic cells failed to damage endothelial monolayers. This has recently been confirmed by Lindner *et al.* [43], who have shown that only irradiated or lipopolysaccharide-treated peripheral blood mononuclear cells induce HUVEC apoptosis, while untreated cells have no effect.

Table 2: *Percentage of Apoptosis in Different Types of Cocultures*

Cells deposited on HUVEC monolayer	HUVEC apoptosis	Percentage of cell adhesion
Mammary epithelial cells		
Normal mammary epithelial cells	98 ± 9%	17.5 ± 4.5
MCF-7	307 ± 83%	80.5 ± 3.6
MDA-MB-231	214 ± 27%	77.8 ± 7.5
T 47 D	342 ± 14%	66.2 ± 7.1
Fibroblastic cells		
Normal fibroblasts	117 ± 13%	54.1 ± 6.4
HT-1080 fibrosarcoma	278 ± 53%	85.5 ± 3.1
Hematopoietic cells		
Normal leukocytes	111 ± 5%	37 ± 1.8
Normal leukocytes on (IL-1β-pretreated HUVEC)	51 ± 22%	54.9 ± 13
Jurkat	76 ± 11%	31.1 ± 3.8
HL-60	120 ± 5%	21.2 ± 5.5

Note. Various normal and tumoral cell types were seeded on HUVEC monolayers. To allow comparison between separate experiments, the apoptotic fractions in endothelial control monolayers were set as 100% and the values after addition of tested cells were expressed as a percentage of the control ± standard deviation. The number of independent experiments was 2 to 5. In the last column, attachment of tested cells to HUVEC monolayers is expressed as a percentage of the radioactivity counted in the total amount of tested cells initially seeded on HUVEC monolayers (± standard deviation) as described under Materials and Methods. Values are means of triplicate measurements in two separate experiments.

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Fig.4: *Agarose gel electrophoresis of DNA. DNA was extracted from HUVEC and MCF-7 cell monocultures pooled just before extraction (lane 2), from cocultures (lane 3), or from cells detached from cocultures (lane 5). As positive control, fresh murine thymocytes were cultured overnight (lane 4) as described under Materials and Methods. Lane 1, molecular weight markers.*

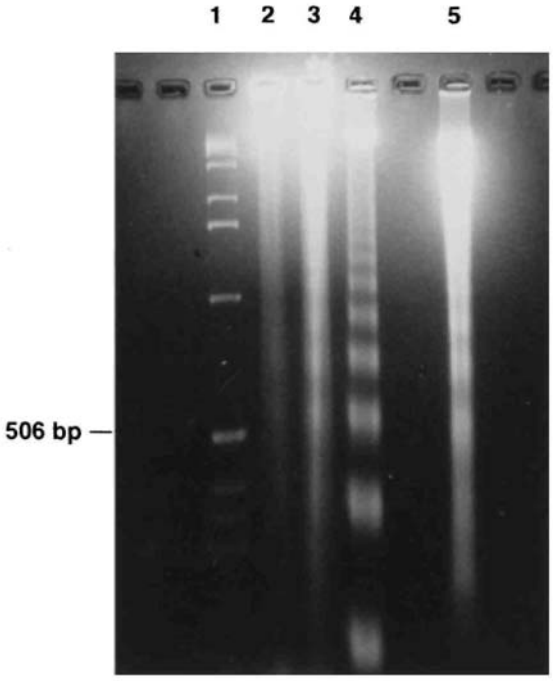
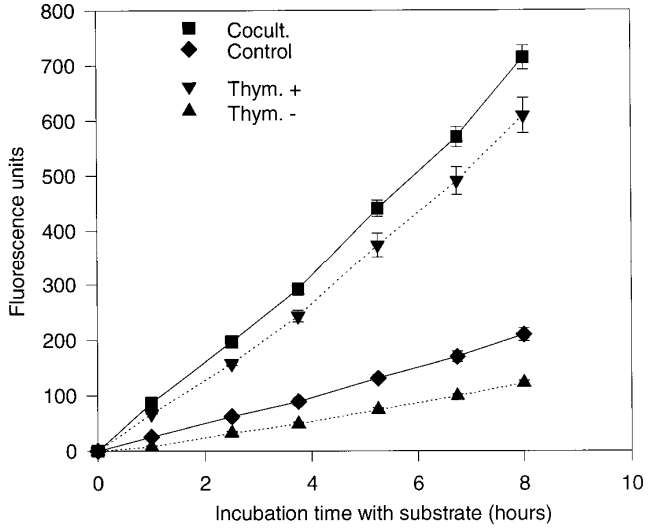


Fig.5: *CPP 32/Caspase-3 activity. Cell extracts (90 µg protein/ ml) were incubated with 50 µM Caspase-3 fluorogenic substrate as described under Materials and Methods. Proteins were extracted from MCF-7-HUVEC coculture (Cocult.), cultured thymocytes as a positive control (Thym. +), separately cultured MCF-7 cells and HUVEC, pooled before extraction (Control), and freshly isolated thymocytes as a negative control (Thym. -). The assay was performed in triplicate. Error bars indicate standard deviations. Results are representative of those obtained from three independent experiments.*



The lack of HUVEC apoptosis induction could not be related to a lack of adhesion to HUVEC monolayers. Indeed, fibroblasts and normal leukocytes, which adhered to HUVEC to the same extent as T47D cells, did not induce endothelial cell retraction or apoptosis. These data suggest that adhesion and apoptosis induction, in our model, are independent events. The ability to damage endothelium seems to be a characteristic of some, if not all, solid tumors, since this was not observed with normal or transformed hematopoietic cells. However, the number of apoptotic HUVEC could not be directly related to the aggressiveness of cancer cells, since more aggressive cells, such as MDA-MB-231, induced the same rate of endothelial apoptosis as did MCF-7 cells. It should be noted that the *in vivo* invasiveness of cells depends not only on their ability to extravasate, but also on their ability to fulfill the other steps of the metastatic process, including migration through host tissue, intravasation, survival in blood flow, extravasation, and invasion of and growth in secondary organs.

Experiments in which endothelial damage was induced in mice by various agents such as antineoplastic drug, high oxygen concentrations, and irradiations provided direct evidence for the promoting effect of vascular damage on hematogenous metastasis formation [for review, see 44]. In this study, we show for the first time that interactions with tumor cells may induce such damage through apoptosis induction. This effect was observed with cells derived from solid tumors but not with normal cells or transformed hematopoietic cells. Our data may lead to a better understanding of the tumor cell extravasation process.

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