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# Dynamic interplay between breast cancer cells and normal endothelium mediates the expression of matrix macromolecules, proteasome activity and functional properties of endothelial cells ☆

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ABSTRACT

*Background:* Breast cancer–endothelium interactions provide regulatory signals facilitating tumor progression. The endothelial cells have so far been mainly viewed in the context of tumor perfusion and relatively little is known regarding the effects of such paracrine interactions on the expression of extracellular matrix (ECM), proteasome activity and properties of endothelial cells.
*Methods:* To address the effects of breast cancer cell (BCC) lines MDA-MB-231 and MCF-7 on the endothelial cells, two cell culture models were utilized; one involves endothelial cell culture in the presence of BCCs-derived conditioned media (CM) and the other co-culture of both cell populations in a Transwell system. Real-time PCR was utilized to evaluate gene expression, an immunofluorescence assay for proteasome activity, and functional assays (migration, adhesion and invasion) and immunofluorescence microscopy for cell integrity and properties.
*Results:* BCC-CM decreases the cell migration of HUVEC. Adhesion and invasion of BCCs are favored by HUVEC and HUVEC-CM. HA levels and the expression of CD44 and HA synthase-2 by HUVEC are substantially upregulated in both cell culture approaches. Adhesion molecules, ICAM-1 and VCAM-1, are also highly upregulated, whereas MT1-MMP and MMP-2 expressions are significantly downregulated in both culture systems. Notably, the expression and activity of the proteasome β5 subunit are increased, especially by the action of MDA-MB-231-CM on HUVEC.
*Conclusions and general significance:* BCCs significantly alter the expression of matrix macromolecules, proteasome activity and functional properties of endothelial cells. Deep understanding of such paracrine interactions will help to design novel drugs targeting breast cancer at the ECM level. This article is part of a Special Issue entitled Matrix-mediated cell behavior and properties.



## 1. Introduction

Cancer is a remarkably diverse disease, characterized by distinctive and complementary hallmark capabilities, involving sustained proliferation or evasion of cell death and immune system destruction, activated invasion and metastasis, induced angiogenesis as well as reprogrammed energy metabolism that coordinate tumor progression [1,2]. The importance of the tumor microenvironment is becoming increasingly appreciated given the fact that tumors are not only self-sufficient moieties but also recruit normal cells of the underlying stroma in favor of tumor progression [3–6]. Metastasis represents a key event of this process that depends on the ability of the cancer cells to grow, degrade the host stroma, migrate into a complex network of macromolecules; extracellular matrix (ECM), adhere, possibly transmigrate through the endothelium into and out of the blood stream and lastly invade the tissue where the metastasis will establish.

In the present study, we focused on a crucial player of this reciprocal relationship between cancer cells and tumor microenvironment, the endothelial cells. The endothelium is highly specialized and its origin varies considerably from tissue to tissue and organ to organ [4,7].

*Abbreviations:* ECM, extracellular matrix; BCC, breast cancer cell; CM, conditioned media; TEM, trans-endothelial migration; GAG, glycosaminoglycan; HS, heparan sulfate; CS, chondroitin sulfate; HA, hyaluronan; UPS, ubiquitin proteasome system; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; BCC-CM, breast cancer cells CM; CAMs, cell adhesion molecules; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular endothelial cell adhesion molecule-1; MMPs, Matrix metalloproteinases; MT1-MMP, membrane-type-1-MMP

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During extra- and intra-vasation, cancer cells undergo critical interactions and establish active contacts with the endothelium that entail remarkable cytoskeletal changes prerequisite for trans-endothelial migration (TEM) into the underlying matrix and stroma [8,9]. Several cancer cell surface molecules have been documented to counteract with molecules on the endothelial cell surface. Among them selectins, integrins, CD44 and the adhesion molecules ICAM, VCAM contribute in that way to the "docking-locking" hypothesis [9].

It is well established that cellular behavior is highly regulated by ECM, and as a consequence cancer progression is dependent on its unique properties, involving alterations in ECM components' structure and/or expression as well as the activity modifications that take place during this process [10,11]. Endothelial glycocalyx represents a negatively charged organized network of ECM macromolecules that lines a healthy vascular endothelium. This scaffold consists of a variety of transmembrane- and membrane-attached molecule proteoglycans (syndecan-1, -2, -4 and glypican-1), as well as cell adhesion molecules (integrins, selectins), inflammatory regulators and adsorbed components (coagulation system) [12,13]. In addition secreted proteoglycans (mimecan, perlecan, and biglycan) reside in the glycocalyx interface or diffuse into the blood stream [14,15]. The most prominent glycosaminoglycan (GAG) incriminated on the surface of endothelial cells is heparan sulfate (HS) accounting for 50%–90% of the total GAG pool, and the rest being composed of chondroitin sulfate (CS) and hyaluronan (HA), with levels depending on cell types [16].

Regardless of its source, the endothelium exerts critical functions in several aspects of cancer biology including tumor progression, angiogenesis, and metastasis. It is documented to regulate angiogenesis in order to provide nutrients, oxygen, and other solutes through the bloodstream to the tumor, supply of paracrine factors recruiting other stromal players like immune cells to the tumor site and modulation of cancer cell dissemination [17,18]. An additional level in the complexity of the interplay between cancer cells and endothelial cells arises from the dynamic interaction of glycocalyx with the local microenvironment as endothelial cells have high rates of continuous metabolic turnover that allow adaptation to changes [19]. Compelling evidence also suggests that the glycocalyx on endothelial cells functions as a matrix barrier. It is well established that tumor cell adhesion to the microvessel wall degrades the endothelial surface glycocalyx layer [20]. Taken all together, cancer cell extravasation is induced by the disruption of this layer by pro-inflammatory cytokines and degrading enzymes produced by cancer cells, thereby influencing vascular endothelial barrier integrity [21–25]. On the other hand, the endothelial glycocalyx composition is able to act itself as a positive or negative modulator of cell adhesion to endothelium. For example, if the size of constituent parts of the endothelial surface layer such as HS proteoglycans and hyaluronan is greater than the size of adhesion molecules, the adherence of cells is prevented [26]. On the other hand, endothelial derived hyaluronan serves as ligand for its adjacent receptor CD44 expressed in cancer cells [27]. Furthermore, endothelial glycocalyx entraps growth factors *via* the glycosaminoglycan chains of proteoglycans, increasing in that way the endothelial permeability [23]. In addition, it reported that through all of these steps of active interaction between cancer cells and the endothelium, both parties undergo critical cytoskeletal changes to facilitate such interplay [28,29].

Proteasome is a major cellular protease complex that regulates non-lysosomal degradation, thus in turn controlling the concentration and turnover of ECM [30]. Several proteasome inhibitors are proposed as novel anti-cancer agents, exerting anti-tumor activity *in vivo*. Apart from its role in cancer cell apoptosis/growth, ubiquitin proteasome system (UPS) has been indicated in several cases directly or indirectly to the dys-function of the endothelial front.

The reciprocal interactions between the endothelium and cancer cells are a complex scenario that is not fully characterized yet. In the present study, we therefore focus on the evaluation of the effects of breast cancer cells on the endothelium microtubules and functional properties, expression of matrix and cell surface effectors (endothelial glycocalyx composition) and proteasome activity.

## 2. Materials and methods

### 2.1. Chemicals and reagents

FBS (fetal bovine serum), DMEM, sodium pyruvate, L-glutamine, penicillin, streptomycin, amphotericin B and gentamycin were all obtained from Biochrom (Berlin, Germany).

### 2.2. Cell cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, USA) and were grown for 2–6 passages in EGM2 culture medium (Lonza) supplemented with 2% fetal bovine serum. The cultures were maintained in an atmosphere of humidified 95% air, 5% $CO_2$ at 37 °C.

Breast cancer cells of human breast adenocarcinoma, MDA-MB-231 (ATCC® HTB-26) and MCF-7 (ATCC® HTB-22) were obtained from the American Tissue Culture Collection. Breast cancer cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1.0 mM sodium pyruvate, and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin sulfate and 2.5 μg/mL amphotericin B). Cells were routinely grown at 37 °C in a humidified atmosphere of 5% (v/v) $CO_2$. Culture medium was changed every 48–72 h. Cells were harvested by trypsinization with 0.05% (w/v) trypsin in PBS containing 0.02% (w/v) $Na_2EDTA$.

### 2.3. Experimental sets

Two experimental approaches were utilized; one involves endothelial cell culture in the presence of breast cancer cell-derived conditioned media (BCC-CM) and the other co-culture of both cell populations in a Transwell system using 0.4 μM microporous membrane (Fig. 1). In some cases the effect on HUVEC CM on breast cancer cells culture was also evaluated. In the CM experimental approach, the role of the produced factors under growing conditions and their effect on the adjacent microenvironment were evaluated. Introducing to our approach the element of cell–cell communication, the co-culture transwell system was utilized where breast cancer cells and HUVEC interact and exchange soluble factors between the microporous membrane.

*HUVEC (BCC-CM approach)*. Six hours before treatments, subconfluent (*ca* 70%) HUVECs were cultured in EGM2 with 0.2% fetal bovine serum. The medium was then changed to EGM2 plus 2% fetal bovine serum supplemented with equal amount of BCC-CM and incubated for 24 h (Fig. 1A).

*Cancer cells (HUVEC-CM approach)*. Six hours before treatments, subconfluent breast cancer cells (*ca* 70%) were cultured in serum free DMEM. The medium was then changed to DMEM plus 2% fetal bovine serum supplemented with equal amount of HUVEC-CM and incubated for 24 h (Fig. 1B).

Conditioned media (BCCs-CM & HUVEC-CM) were generated from confluent cell monolayers by 24 h of culture in the appropriate culture medium. Cells and debris were removed by centrifugation (5 min, 500 ×*g*), and media were aliquoted and stored at −80 °C. In each case the culture media of the adjacent utilized CM media served as negative control of the experiment.

*Transwell approach*. HUVEC (lower chamber) and breast cancer cells (upper chamber) are co-cultured in EGM2 plus 2% and DMEM plus 2%, respectively using a transwell insert with 0.4 μM microporous membrane. Control HUVEC were cultured in the presence of culture media of BCCs in the upper chamber. Before the incubation cell monolayers were serum starved like the CM approach (Fig. 1C).

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