Objective: In vitro assessment of ICAM-1, VCAM-1 and P-selectin expression on endothelial cells cocultured with c-erbB2-positive (SK-BR-3) and c-erbB2-negative (MCF-7) breast carcinoma cell lines.

Material and Methods: The expression of adhesion molecules was determined by immunofluorescence, and measured by a laser scanning cytometer.

Results: Endothelial cells cocultured with c-erbB2-negative breast carcinoma cell line – as compared to endothelial cells cultured without carcinoma cells – demonstrated an increase in the expression of VCAM-1 and P-selectin, a decrease in the expression of ICAM-1, and an increase of the maximal pixel intensity of ICAM-1, VCAM-1 and P-selectin orange fluorescence. A higher increase of the expression and maximal pixel intensity of ICAM-1, VCAM-1 and P-selectin was observed in endothelial cells cocultured with c-erbB2-positive breast carcinoma cells as compared to endothelial cells cocultured with c-erbB2-negative cell line.

Conclusions: An increase in the expression of adhesion molecules on endothelial cells cocultured with c-erbB2-positive breast carcinoma cells may be responsible for the increased adhesion of tumor cells to endothelium and the increased invasiveness of c-erbB2-positive breast carcinomas.

Introduction

The C-ERBB2 (HER-2/neu) protooncogene encodes a 185 kDa transmembrane receptor protein with intrinsic tyrosine kinase activity. Amplification or overexpression of C-ERBB2 has been detected in 20-30% of invasive breast carcinomas and is associated with adverse prognostic factors such as higher histological grade, tumor necrosis, high mitotic activity, lack of estrogen and progesterone receptors, p53 and vimentin expression, aneuploidy, metastases to lymph nodes, a shorter overall survival and disease-free survival time; moreover C-ERBB2 is a predictive factor with regard to monoclonal antibody therapy with Trastuzumab [9, 13, 19, 28, 29, 31, 32].

Cancer metastasis is a multistep process that involves local tumor growth, invasion of the extracellular matrix followed by vascular dissemination and secondary growth at a distant site. The ability of cancer cells to interact with the endothelium via adhesion molecules such as VCAM-1 (Vascular Cell Adhesion Molecule-1, CD54), ICAM-1 (Intercellular Adhesion Molecule-1, CD106) and P-selectin (CD62P) appears to be a prerequisite for the development of distant metastasis [12, 22]. The ICAM-1 molecule is present on the surface of endothelial cells, leukocytes, fibroblasts, some non-neoplastic epithelial cells, as well as on cancer cells [1]. VCAM-1 is noted on the surface of macrophages, dendritic and muscle cells, and on endothelial cells [1]. P-selectin is found in the membranes of Weibel-Palade bodies in endothelial cells, as well as in blood platelet granules [4].

In c-erbB2 positive breast cancers C-ERBB2 overexpression is associated with increased tumor progression and metastasis; however, the exact mechanism by which C-ERBB2 regulates more aggressive phenotype is not fully understood [13, 18, 20, 23, 31]. Since the interactions
between cancer cells and endothelial cells in vascular dissemination and homing of tumor cells also remain poorly defined, we decided to study in vitro the expression of adhesion molecules: ICAM-1, VCAM-1 and P-selectin on endothelial cells cocultured with c-erbB2-positive (SK-BR-3) and c-erbB2-negative (MCF-7) breast carcinoma cell lines [11, 12, 21, 24].

Material and Methods

Cell cultures

Endothelial cells

Endothelial cells were isolated from human umbilical cord veins that originated from healthy women. To remove blood, the umbilical cord vein was rinsed with phosphate buffered saline (PBS, Sigma-Aldrich Corporation, ST. Louis, Missouri, USA). Subsequently, in order to detach endothelial cells from the basement membrane, a 0.1% Ia collagenase solution (Biochrom AG, Berlin) with the activity of 132 U/ml and temperature of 37°C was injected into the vein for 10 minutes. The cells detached by collagenase were transferred by rinsing to a test tube containing 5 ml of fetal bovine serum (FBS, Invitrogen Corporation, Paisley, Scotland, UK) and洗涤 in a 20% solution of FBS in PBS, and the cells were suspended in 10 ml of Medium 199 containing 20% FBS. The medium solution was enriched with antibiotics: 50μl penicillin with streptomycin (Invitrogen Corporation, Paisley, Scotland, UK), 100 U/ml amphotericin B (Sigma-Aldrich Corporation, ST. Louis, Missouri, USA) and 100 μl amphotericin B (Sigma-Aldrich Corporation, ST. Louis, Missouri, USA). Cell suspension was transferred to T-25 culture bottles (Nunc A/S, Roskilde, Denmark), previously coated with endothelial cell attachment factor (ECAF, Sigma-Aldrich Corporation, ST. Louis, Missouri, USA). The cells were incubated in a humid chamber, then rinsed in 2 ml PBS cooled to +4°C. The cells were cultured in T-25 culture bottles in McCoy’s medium (American Type Culture Collection, Manassas, VA, USA) enriched with 10% FBS, with 100 U/ml penicillin and streptomycin in the same conditions as endothelial cells. The experiments were carried out on first and second passage cells. After 4 days, the cells were detached from the surface of the culture bottle by trypsin, using the above described method. Following double rinsing and centrifuging (772 rpm, 5 minutes) in solution of FBS in PBS, the suspensions of 2 x 10⁶ cells in 100 μl were transferred to culture slides, where endothelial cells had been already growing. The endothelial cells were cultured in the presence of the cancer cells for 24 hours. Each experiment was repeated thrice. The expression of ICAM-1, VCAM-1 and P-selectin was studied in cultures of endothelial cells grown without cancer cells (control cultures), cocultures of endothelial cells and MCF-7 breast carcinoma cell line and cocultures of endothelial cells and SK-BR-3 breast carcinoma cell line.

Detection of adhesion molecules expression by immunofluorescence.

Following a 24-hour cocultivation of endothelial cells and breast carcinoma cells, the medium was removed and the cells were rinsed with 2 ml PBS cooled to +4°C. The cells were incubated with mouse anti-human ICAM-1, VCAM-1 and P-selectin antibodies conjugated with PE (phycoerythrin) (BD Pharmingen, San Diego, USA, sub-sequent catalog numbers 555511, 555647, 555524). To identify endothelial cells, anti-PECAM-1 antibody (Platelet-Endothelial Cell Adhesion Molecule-1, CD31) conjugated with FITC (fluorescein) (BD Pharmingen, San Diego, USA, catalog number 555445) was used. PECAM-1 is a protein present on endothelial cells of all types of blood vessels and may serve as a marker of such cells [26, 30].

The cells were incubated with the antibodies for 30 minutes at +4°C in a humid chamber, then rinsed in 2 ml PBS, covered with a cover glass, and the fluorescence parameters of individual cells were measured by laser scanning cytometer (LSC, CompuCyte, Cambridge, MA, USA).
In vitro Assessment of Adhesion Molecules Expression by ...

... equipped with the WinCyte software. In each slide, at least 5,000 cells were measured.

**Analysis of cellular fluorescence using a laser scanning cytometer.**

The cell contouring parameter was set on FITC green fluorescence associated with an antibody that binds the PECAM molecule present on all endothelial cells. FITC green fluorescence (525 nm emission) and PE orange fluorescence (575 nm emission) excited by an argon laser (488 nm) were recorded by LSC and changes of the integrated orange fluorescence (Orange Integral) and the maximal pixel intensity of orange fluorescence (Orange Max Pixel) associated with ICAM-1, VCAM-1 and P-selectin were measured. The integrated fluorescence is the sum of fluorescence of all the pixels within the integrated fluorescence contour. The maximal fluorescence pixel is the highest fluorescence intensity value for a single pixel within the integrated fluorescence contour. Their values range between 0 to 8,000,000 and 0 to 16,400 respectively of relative units for a single fluorescence measurement.

The principles of analyzing fluorescence parameters of endothelial cell adhesion molecules are presented in Fig. 1. Based on the dot-plot illustrating the relation of the endothelial cells area (Area Green) and the maximal pixel of PECAM – bound FITC green fluorescence (Green Max Pixel) cell population for further analysis was determined (Graph A). In region selected on graph A integrated VCAM-1 - bound orange fluorescence (Orange Integral, Graph B, black histogram in grey box) and the maximal pixel of VCAM-1 – bound orange fluorescence (Orange Max Pixel, Graph C, black histogram in grey box) were evaluated. The integrated orange fluorescence and the maximal pixel of orange fluorescence were measured above the control autofluorescence level (white histograms), or – in other words – above the fluorescence level of non-stained endothelial cells from the control cultures [14]. The same principles of analysis were employed to assess the integrated orange fluorescence and the maximal pixel of orange fluorescence emitted by ICAM-1 and P-selectin.

**Statistical methods**

The arithmetic mean values and the standard deviation of error (SDE) were calculated for each investigated parameter. To evaluate the distribution of the mean values of the integrated orange fluorescence and maximal pixel of orange fluorescence in endothelial cells, the Kolmogorov-Smirnov test was used. Since the investigated parameters differed from the normal distribution and the number of cells exceeded 2,000, to evaluate the differences between the investigated parameters in endothelial cells cultured without or with the MCF-7 and SK-BR-3 cell lines the Kolmogorov-Smirnov test was also used. P values less than 0.005 was considered statistically significant.

**Results**

Changes in the integrated orange fluorescence and maximal pixel of orange fluorescence emitted by ICAM-1, VCAM-1 and P-selectin in endothelial cells cultured with and without breast carcinoma cells are presented in Fig. 2 and Fig. 3.

An increase in the integrated orange fluorescence of VCAM-1 (p<0.001) and P-selectin (p<0.001), and a
drop in the integrated orange fluorescence of ICAM-1 (p < 0.001) in endothelial cells cocultured with MCF breast carcinoma cell line as compared to the control cultures was noticed. In endothelial cells cocultured with SK-BR-3 breast carcinoma cell line an increase in the integrated orange fluorescence related to all measured adhesion molecules, i.e. ICAM-1 (p < 0.001), VCAM-1 (p < 0.001) and P-selectin (p < 0.001) as compared to the control cultures was observed.

The analysis of the maximal pixel of orange fluorescence of adhesion molecules demonstrated an increase of ICAM-1 (p < 0.001), VCAM-1 (p < 0.001) and P-selectin (p < 0.001) in endothelial cells cocultured with MCF-7 breast cancer cell line as compared to the control cultures, as well as an increase of the maximal pixel of orange fluorescence related to ICAM-1 (p < 0.001), VCAM-1 (p < 0.001) and P-selectin (p < 0.001) in endothelial cells cocultured with SK-BR-3 breast cancer cell line.

Further, the increase of the maximal pixel of orange fluorescence of ICAM-1 (0.001 < p < 0.005), VCAM-1 (p < 0.001) and P-selectin (p < 0.001) was higher in cocultures of endothelial cells and SK-BR-3 breast cancer cell line as compared to endothelial cells cocultured with MCF-7 breast cancer cell line (1.09 vs. 1.06 vs. 1.57 respectively; data not shown).

Discussion

Breast cancer metastases rely mostly on lymphatic and blood vessel systems by which cancer cells can spread widely into regional lymph nodes or distant sites. To date, many factors have been reported to be critically involved in regulating the blood vessel formation in tumor development [18, 21]. Little is known about how cancer cells interact with endothelial cells. These interactions are required for adhesion of cancer cells to the surface of endothelial cells and subsequently transendothelial migration of cancer cells to extravascular space that leads to the growth at the new site [6, 12, 21, 24].

Our study revealed an increase of VCAM-1 and P-selectin expression in endothelial cells cocultured with MCF-7 cells as well as with SK-BR-3 cells and an increase of ICAM-1 expression in endothelial cells cocultured with SK-BR-3 cells.

The mechanism of the effect of carcinoma cells on the increased expression of adhesion molecules on endothelial cell surface is related to cytokines and mitogens. Cytokines such as VEGF, bFGF, TGFβ, TNFα and interleukins released by cancer cells activate endothelial cells and induce an increase in the expression of adhesion molecules by binding to appropriate receptors on endothelial cells and activation of transcription factors NF-κB and activator protein-1 (AP-1) [14, 15]. NF-κB is a heterodimer consisting of two subunits p50/p65 found in the cytoplasm in an inactive form combined with IκB protein [17]. As the effect of cytokines, IκB is degraded and active NF-κB is translocated to the cell nucleus [14]. Transcription factor AP-1 is a multiprotein complex composed of the products of C-JUN and C-FOS proto-oncogenes [15]. In the nucleus, NF-κB and AP-1 activate transcription not only of the cytokine genes, e.g. TNFα, IL-1, IL-6, IL-8, INFγ, but also the E-selectin, P-selectin, VCAM-1 and ICAM-1 genes, as well as genes encoding proteins exhibiting anti-apoptotic activity and regulating the mechanisms of multi-drug resistance [3, 15, 30, 32].

Interestingly, endothelial cells cocultured with MCF-7 carcinoma cells demonstrated a drop of the integrated orange fluorescence of ICAM-1 as compared to the control endothelial cells but this drop of ICAM-1 fluorescence was associated with an 1.21-fold increase of the maximal pixel ICAM-1 - associated orange fluorescence in the same cocultures. Similar observation has been reported by Fox et al. who found an increased expression of VCAM-1, ICAM-3, P and E-selectin, but decreased expression of ICAM-1 and ICAM-2 on the endothelium of tumor associated vessels in invasive breast carcinomas [8]. Down-regulation of ICAM-1 on tumor-infiltrating endothelial cells has also been ob-
served in renal cell carcinoma. The effect was specific for bFGF only since other angiogenic factors such as VEGF, TGFβ and IL-8 did not affect ICAM-1 expression [10].

The measurement of the maximal pixel by LSC allows detection of local fluorochrome aggregation in the cell [7]. It has been demonstrated that the measurement of the maximal pixel of fluorescence associated with anti-Bax antibody can detect its translocation and accumulation in the mitochondria of cells undergoing apoptosis [2]. Similarly, the measurement of the maximal pixel of red fluorescence emitted by cells stained with fluorochrome, specifically binding DNA, i.e. with propidium iodide in the presence of RNase, allows for detection of changes in nuclear chromatin condensation and for discrimination between apoptotic and non-apoptotic cells, mitotic and interphase cells, as well as for determining white blood cell subpopulations in peripheral blood, i.e. lymphocytes, monocytes and neutrophils [7]. The increase of the maximal pixel of orange fluorescence emitted by ICAM-1, which was accompanied by a drop in the integrated value of orange fluorescence, may point to an increased aggregation of ICAM-1 in endothelial cells cocultured with MCF-7 line cancer cells. Possibly, the observed aggregation of ICAM-1 is a result of cup-like structures formation, that may play a role in transendothelial migration of cancer cells [5, 6].

One of the important consequences of high levels of c-erbB2 in c-erbB2-positive breast cancer cells is the excessive secretion of VEGF [16, 18]. The observed association between the overexpression of C-ERBB2 and higher VEGF expression indicates that c-erbB2 is involved in the increased angiogenesis in human breast cancers. The presumed molecular mechanism of the association between C-ERBB2 overexpression and VEGF expression involves VEGF gene transcription mediated by hypoxia-inducible factor (HIF-1), which is a transcription factor [16]. It is not excluded that some additional, not fully defined pathways of adhesion molecule stimulation may play a role in the more intense expression of endothelial adhesion molecules in the presence of c-erbB2 positive breast cancer cells. It has also been demonstrated that VEGF in rat primary brain microvascular endothelial cells up-regulates the expression of ICAM-1 through a pathway that includes phosphatidylinositol 3 OH-kinase, AKT, and nitric oxide [25].

The present results indicate that c-erbB2-positive breast cancer cells induce the expression of ICAM-1, VCAM-1 and P-selectin on endothelial cells to a greater extent than c-erbB2-negative breast cancer cells. The integrated orange fluorescence emitted by ICAM-1, VCAM-1 and P-selectin was 1.27-fold, 1.4-fold and 1.51-fold higher, respectively, in endothelial cells cocultured with SK-BR-3 line cancer cells as compared to cocultures with MCF-7 line cancer cells.

We conclude that an increase in the expression of adhesion molecules on the surface of endothelial cells cocultured with c-erbB2-positive breast cancer cells may be responsible for the increased adhesion of cancer cells to endothelial cells and the higher invasiveness of c-erbB2-positive breast carcinomas. Cocultures of endothelial cells with cancer cells on special microscopic culture slides and evaluation in situ of the expression of various proteins by means of LSC may serve as a model for investigating the effect of different drugs on the expression of selected proteins in endothelial cells cocultured with cancer cells collected from individual patients with breast carcinomas [19, 20, 27].

References


