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Breast Cancer Conditioned Media Modulate the Expression of Immune Checkpoints and Adhesion Molecules in Endothelial Cells

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Abstract

Objective: Breast cancer is commonly originated from the cells in the milk ducts or in the lobules that supply milk to the ducts. During cancer development, many actors, including cellular factors such as endothelial and immune cells, and structural factors such as integrins and extracellular matrix elements have gained importance in the tumor microenvironment. Endothelial cells (ECs) not only implicate in cancer growth and metastasis processes, but also are effective in immune cells' modulations. In the present study, we investigated the effect of secretome released by breast carcinoma cell lines on endothelial expression of immune checkpoints and adhesion molecules.

Materials and Methods: In this study, the expression levels of some immune checkpoints, including lymphocyte-activation-protein 3 (LAG-3), V-domain Ig suppressor of T-cell activation (VISTA), Indoleamine-2,3-dioxygenase (IDO), IDO-2, programmed cell death 1 ligand (PD-L1), PD-L2, Cytotoxic T-lymphocyte associated protein 4, (CTLA-4), and some adhesion molecules, including E-cadherin, VE-cadherin, E-selectin, platelet EC adhesion molecule-1 (PECAM-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), were measured using quantitative real-time polymerase chain reaction in HUVEC ECs preincubated with the conditioned media (CM) collected from normal (CRL-4010) and carcinoma cells (CRL-2329 and MDA-MB-231). Besides, endothelial proliferation and migration were also examined.

Results: CRL-2329-CM was found to increase cell proliferation at the end of 48 h but not 24 h, compared to the control (CRL-4010-CM) group ($p=0.0052$). In addition, both cancer cell-CMs were revealed to induce migration ($p<0.001$). VE-cadherin, ICAM-1, PECAM-1, VCAM-1, and E-selectin gene expressions were found to be increased significantly in the 48 h groups ($p<0.001$). The expression levels of PD-L1 ($p=0.0028$ and $p<0.001$), PD-L2 ($p=0.0216$ and $p<0.001$), IDO-1 ($p<0.001$), LAG3 ($p<0.001$ only for MDA-MB CM group), VISTA ($p<0.001$) genes in 48 h groups, and PD-L2 ($p=0.0084$ and $p=0.0045$) gene in 24 h groups, were significantly increased. However, we found that there were no expressions of IDO-2, CTLA-4 and E-cadherin genes in ECs.

Conclusion: This study has shown that secretome produced by breast carcinoma cell lines may promote endothelial proliferation and migration and support metastasis. Also, secretome can be effective on immune modulation.

Keywords: Conditioned media, breast cancer, HUVEC, immune checkpoints, adhesion molecules

Introduction

Breast cancer is the most common and diagnosed type of cancer in women, and also is the second leading cause of cancer-related deaths among them (1). During cancer development, growth and metastasis, several cell types interact each other and various mediators such as cytokines, integrins, growth factors and matrix metalloproteinases play critical roles in the tumor microenvironment (2,3). Different cell types in this environment such as immune cells (e.g. cytotoxic and regulatory T-cells) and endothelial cells (ECs) are also involved in the cancer growth process (4-6). ECs can regulate the feeding, intravasation and extravasation of tumor cells (3,6). It is well known that one of the typical features of ECs is the expression of a variety of adhesion molecules such as E-selectin, integrins, VE-cadherin and some members of immunoglobulin

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superfamily, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), platelet EC adhesion molecule-1 (PECAM-1), which all allow leukocytes to adhere, and to migrate to an inflammatory site (2,7). Also, adhesion molecules on ECs, for example ICAM-1, are involved in adhesion, intravasation and extravasation to migrate to the distant organ sites of cancer cells (8,9).

Cytotoxic activity of immune cells which are responsible for the immune surveillance of cancer and for the regression of tumor growth can be inhibited by cellular ligands and cytokines in tumor microenvironments (10). Among them, programmed cell death 1 ligand (PD-L1) and PD-L2, known as programmed cell death ligands, were shown to be two of the most effective ligands expressed on the surface of a variety of cells (e.g. cancer cells and antigen presenting cells) (11,12). PD-L1 is known as a negative regulatory molecule that mainly regulates T-cell activation and maintains antigen tolerance (11,13). Presentation of various ligands by ECs to T-cells mainly provide stimulatory signals that promote activation of them (13). However, it was reported that interferon-γ stimulation and consecutive tumor necrosis factor-alpha treatment positively regulated the endothelial expression of PD-L1 and PD-L2, and down-regulated the activation of $CD8^+$ T lymphocytes (13).

Another important target in cancer immunotherapy is the rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO) (14). This enzyme catalyzes the conversion of tryptophan amino acid into kynurenine. IDO-mediated depletion of tryptophan and increase of the catabolic product kynurenine in tumor microenvironment inhibit the growth and survival of T lymphocytes, thus allowing cancer cells to evade the immune system (14).

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4), V-domain Ig suppressor of T-cell activation (VISTA) and lymphocyte-activation-protein 3 (LAG-3), which are among the other immune checkpoint genes, have negative regulatory effects against T-cells activation, leading to dysfunction and growth suppression of T-cells (15,16). Treatment of monoclonal antibodies against VISTA immune suppressive ligand was reported to increase cell number and intratumoral function of T-cell in murine tumor models, supporting anti-tumoral immunity (17,18).

As described above, PECAM-1, E-cadherin, VCAM-1, ICAM-1, E-selectin and VE-cadherin adhesion molecules, and LAG3, VISTA, CTLA-4, PD-L1, PD-L2, IDO-1 and IDO-2 immune checkpoints have together displayed crucial roles in cell proliferation and metastasis. In this study, to better understand the roles of ECs under media secreted from cancer cells, we examined the roles of culture medium of breast cancer cell lines and normal conditioned media (CM) on EC proliferation, migration, and also, we investigated the expression status of these adhesion ligands and immune checkpoints.

Materials and Methods

Reagents for Cell Culture

The following materials purchased for cell culture experiments are listed with catalog number: Human umbilical vein ECs (Lonza C2519A), breast cancer cell lines (ATCC-MDA-MB-231 and ATCC-CRL-2329) and normal breast epithelial cells (ATCC-CRL-4010), EndoGrow EC Growth Medium (Diagnovum D501), DMEM (Thermo Fisher 11965084), MTT (Sigma M5655).

Cell Culture

HUVECs and breast cell lines were grown in EndoGRO-MV Complete Culture Medium and DMEM medium, respectively. Cancer cell medium was supplemented with 10% fetal bovine serum (FBS, Diagnovum D151) and 1% penicillin-streptomycin antibiotic cocktail (Diagnovum D910). Conditioned medium plus DMEM containing 2% FBS was applied to the experimental groups at a ratio of 1:1. Endothelial and carcinoma cells were incubated under 5% CO_2 and humidified atmosphere at 37°C.

Experimental groups were as follows:

1. Control Group: ECs grown in CRL-4010 (normal breast cells) CM, 2. CRL-2329 Group: ECs grown in CRL-2329 (mammary epithelial ductal carcinoma cells) CM, 3. MDA-MB-231 Group: ECs grown in MDA-MB-231 (mammary epithelial adenocarcinoma cells) CM.

Conditioned Media (CM) Preparation

After CRL-4010, CRL-2329 and MDA-MB-231 cells were cultured at a confluency of 80-90% in suitable conditions described above, their media were collected into tubes. The collected media were centrifuged at 3.000 g for 10 minutes at 4°C. Obtained supernatants were filtered through a 0.22 µm syringe filter and stored at -80°C until use. Conditioned medium was applied to the ECs at a ratio of 1:1 v/v DMEM containing 2% FBS:CM for 24 and 48 hours.

Cell Proliferation Assay

After applied with the CM for 24 hours and 48 hours, ECs' viability was assayed using tetrazolium MTT technique. Tetrazolium stock solution in HBSS was prepared as 5 mg/mL concentration and diluted 10-fold to make a working solution. ECs grown under suitable conditions in T25 culture dishes were trypsinized and plated into 24-well petri dishes as 5 x 10^4 cells/well. When ECs reached to approximately 70-80% confluency, cells were treated with the serum starved medium containing

2% FBS overnight. Then medium was exchanged with the CM and incubated for 24 and 48 hours. When incubation period was completed, cell culture media were discarded and 500 µL of tetrazolium working solution in HBSS was supplied into each well and incubated for 3 h at 37°C. Formazan crystals in ECs were solubilized with 500 µL of isopropanol solution containing 0.04 M HCl by pipetting up and down. The absorbance of solution was measured at 570 nm by using a spectrophotometric microplate reader (Thermo Scientific Multiskan GO).

Cell Migration Assay

To analyze EC migration, we used cell scratching method named as "wound healing assay" by which distance between cells after movement of cells across the scratch was measured. After incubated under serum-starved conditions overnight in 12-well petri, confluent cells were scratched across petri dish diameter using a sterile pipette tip of 1000 µL. The media in the wells was withdrawn and washed with HBSS. After washing, ECs were treated with CM for approximately 10 hours. After about 10 hours, cell images were taken under 4x magnification using an inverted microscope (Leica DFC450) and its software. To measure the gap width as pixel, we analyzed the distances of ten different fields in the images via ImageJ software, and calculated the mean of data.

Relative Gene Expression Assay by qRT-PCR

Cells in a T25 culture flask were trypsinized, counted, and seeded into 6-well petri dishes as $3x10^5$ cells/well. When the cells reached 80-85% confluency, the old media were replaced with fresh 1:1 CM/DMEM, containing 2% FBS. Cells were taken from the incubator approximately 24 and 48 hours later and total RNA was immediately purified using a commercial kit (Biobasic EZ-10 Spin Column Total RNA Miniprep Kit). Quantification and purity of the obtained RNAs were determined by reading at a wavelength of 260 and 280 nm with a nanodrop spectrophotometer. Total RNA was converted to cDNA by using a commercial kit (Abm Good Onescript cDNA Synthesis kit) and a thermal cycler according to the manufacturer's instructions.

To evaluate the relative gene expression levels of E-cadherin, VCAM-1, E-selectin, ICAM-1, PECAM-1, and VE-cadherin adhesion molecules, and LAG3, VISTA, CTLA-4 PD-L1, PD-L2, IDO-1 and IDO-2 immune checkpoints, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using ABM Brightgreen 2x qPCR Mastermix Kit and Qiagen qRT-PCR Rotor-Gene Q instrument. GAPDH was assayed as a house-keeping gene.

5′ to 3′ Primers (Forward and Reverse, respectively) for the Genes Were as Follows:

GAPDH: GTCTCCTCTGACTTCAACAGCG / ACCACCCTGTTGCTGTAGCCAA; ICAM-1: AGCGGCTGACGTGTGCAGTAAT / TCTGAGACCTCTGGCTTCGTCA; VCAM-1: GATTCTGTGCCCACAGTAAGGC / TGGTCACAGAGCCACCTTCTTG; E-Selectin: TCAAGGGCAGTGGACACAGCAA / GGAAACTGCCAGAAGCACTAGG;

PECAM-1: AAGTGGAGTCCAGCCGCATATC / ATGGAGCAGGACAGGTTCAGTC;

VE-cadherin: GAAGCCTCTGATTGGCACAGTG / TTTTGTGACTCGGAAGAACTGGC;

PD-L1: TGCCGACTACAAGCGAATTACTG / CTGCTTGTCCAGATGACTTCGG; PD-L2: CTCGTTCCACATACCTCAAGTCC / CTGGAACCTTTAGGATGTGAGTG;

IDO-1: GCCTGATCTCATAGAGTCTGGC / TGCATCCCAGAACTAGACGTGC;

IDO-2: GTTATGTCTGGCAGGAAGGAGAG / GTCCAGTTCGTCAGCACCAAGT;

VISTA: AGATGCACCATCCAACTGTGTGG / AGGCAGAGGATTCCTACGATGC;

LAG-3: GCAGTGTACTTCACAGAGCTGTC/ AAGCCAAAGGCTCCAGTCACCA

Real-time PCR was performed under the following reaction conditions:

1. First denaturing step at 95°C for 10 min, 2. PCR cycle (40 cycles) at 95°C for 15 sec, at 60°C for 60 sec and eventually single fluorescence reading for each cycle. Besides, melting curve analysis was added to the software program as follows: 1. Denaturation step of PCR products at 95°C for 30 sec, 2. Cooling step at 55°C for 60 sec. 3. Increasing the temperature step by step from 55°C to 95°C. In this step, temperature level was increased by 0.2°C per second and fluorescence signals were continuously received. All experiments were performed in duplicate and three times. Relative comparisons of mRNA expressions were calculated using the formula known as $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

To evaluate the statistical analysis of data obtained from the experiments, GraphPad Prism 5.01 (San Diego, CA, USA) program was used. Data were evaluated by repetitions of experiments performed at least 3 times. Descriptive statistics of the obtained data were made with Column Statistics. The results from this study were indicated as mean ± standard deviation. To analyze whether

the data conformed a normal distribution, the Shapiro-Wilk normality test was performed. Comparisons of the results between experimental groups, including cell migration, cell proliferation and gene expression assays, were made using the one-way ANOVA test. Besides, as post-hoc tests, the Tukey multiple comparison test was applied and p values less than 0.05 were considered as statistically significant.

Results

Breast Cancer CM Types Show Diverse Effects on Cell Proliferation

Our study has shown that CRL-2329 breast cancer CM significantly increase cell proliferation in the 48-hour subgroup (p=0.0052), but not in the 24-hour subgroup compared to control (Figure 1). Although the treatment of ECs with CM collected from the MDA-MB-231 adenocarcinoma cancer cell line for 24 h reduced cell proliferation (p=0.0018), these effects were reversed by 48 hours of the treatment. Moreover, ECs incubated with CRL-2329-CM exhibited more increase on cell proliferation than that of MDA-MB-231-CM (p=0.0092) (Figure 1).

Breast Cancer Conditioned Medium Promotes Cell Migration

In this study, for cell migration named as wound healing assay, we observed the capacity of cells to fill the scratched gap by using inverted microscope. In order to examine the effect of CM on ECs, the images of the experimental wells under the microscope were examined at certain hour intervals and their closure was compared after about 10 hours. According to the migration findings, it was observed that the cell migration in CRL-2329-CM groups (Figure 2, Figure 3B-B′) significantly increased as compared to the control (Figure 2, Figure 3A-A′) and MDA-MB-231-CM groups (Figure 2, Figure 3C-C′) (p<0.001). In addition, EC migration was also found to be promoted in the MDA-MB-231 group (p<0.001).

Breast Cancer Conditioned Medium Alters the Expression Profiles of Adhesion Molecule Genes Except for E-cadherin

According to data obtained by qRT-PCR experiments, it was found that the expression levels of all genes of adhesion molecules in 48 hour-treated cells were higher than in 24 hours. *ICAM-1* gene expression in 48-hour groups was elevated dramatically 2.6-fold and 3.9-fold by the treatment with CRL-2329-CM and MDA-MB-231-CM, respectively, when compared to that in control groups (p<0.001). Difference between the cancer CM-groups was also significant (p<0.001) (Figure 4). The present study also revealed that *VCAM-1* gene expression levels significantly increased in 24-hour and 48-hour groups treated with both CRL-2329-CM (2.76-fold and 4.15-fold, respectively, p<0.001) and MDA-MB-231-CM (1.65-fold and 2.84-fold, respectively, p<0.001) groups (Figure 4). Similar to *VCAM-1* gene expression profile, another adhesion related E-selectin gene expression level was found to be remarkably increased in ECs treated with CRL-2329-CM and MDA-MB-231-CM for 24 and 48 hours (2.83-fold, 3.46-fold and 2.37-fold, 2.69-fold, respectively) (p<0.001 for all comparisons), (Figure 4). Regarding to *PECAM-1*, we found that gene expression levels decreased in response to CMs in the 24-hour groups (p=0.0439 and p=0.0179), but markedly increased in the 48-hour groups (p<0.001 in

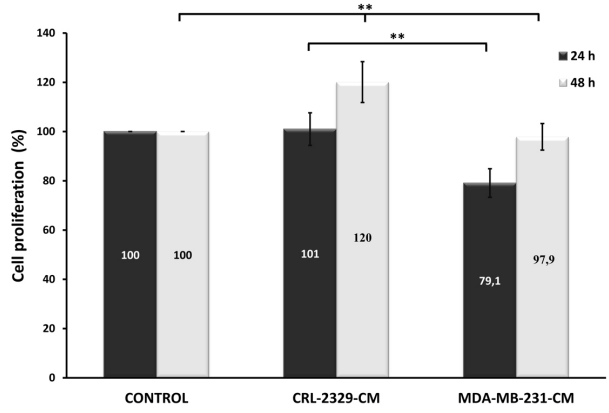

Figure 1. The effect of different breast cancer conditioned media (CRL-2329-CM and MDA-MB-231-CM) on endothelial cell proliferation for 24 h and 48 h. Cells were applied to tetrazolium salt solution and measured calorimetrically at 570 nm. Results were normalized to the corresponding controls (CRL-4010-CM). Data were presented as the mean ± standard deviation of three different experiments and shown as error bars. Significance between the groups was shown as asterisk. **p=0.0018 for C vs. MDA-MB-231-CM and p=0.0092 for CRL-2329-CM vs. MDA-MB-231-CM in 24 h groups; p=0.0052 for C vs. CRL-2329-CM and p=0.0054 for CRL-2329-CM vs. MDA-MB-231-CM in 48 h groups.

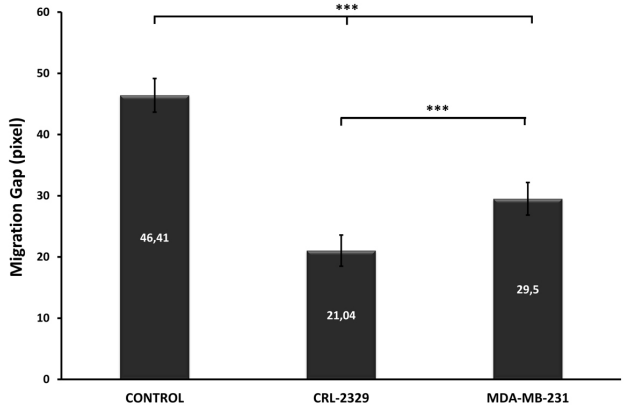

Figure 2. Effects of breast cancer-CM treatments on endothelial cell migration. Bar graphs show the final distance of the cells migrating both sides of the gap in control (CRL-4010-CM), CRL-2329-CM and MDA-MB-231-CM groups. Data represent the mean ± standard deviation of three different experiments and shown as error bars. For each experiment, pixel values of ten different area in the scratched line were averaged. Significance between the groups was shown as asterisk, ***p<0.001.

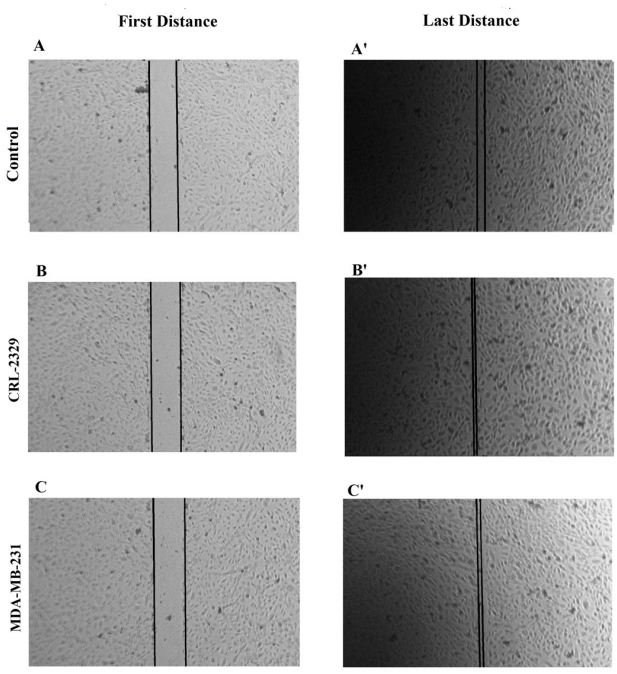

Figure 3. Microphotographs of wound healing assay used for cell migration. While A: Control (CRL-4010-CM), B: CRL-2329-CM, and C: MDA-MB-231-CM show initial scratched gap, A', B' and C' indicates the closure of the corresponding gap.

both groups) (Figure 4). Similarly, although VE-cadherin gene expression levels were interestingly decreased in both the CRL-2329-CM and MDA-MB-231-CM-treated 24-hour groups (0.5-fold and 0.4-fold, respectively), they were increased dramatically in the 48-hour groups (2.08-fold and 2.85-fold, respectively) compared to that in controls ($p<0.001$ for all comparisons) (Figure 4). Since the fluorescent signal appeared after the 36-37th cycle in the PCR reaction, it was evaluated that there was no clear E-cadherin gene expression.

When the changes in adhesion-related gene expression levels are considered in general, it is noteworthy that the increases in ICAM-1, PECAM-1 and VE-cadherin gene expression levels were higher in EC cells treated with MDA-MB-231-CM.

Breast Cancer Conditioned Medium Modulates Immune Checkpoint Genes Except for CTLA-4 and IDO-2 in Endothelial Cells

We evaluated endothelial qRT-PCR expressions of immune checkpoint molecules, PD-L1, PD-L2, IDO-1, VISTA, and LAG3 and found a significant increase especially in MDA-MB-231-CM applied groups for 48 hours (2.37-fold, 1.94-fold, 2.05-fold, 2.61-fold, 3.71-fold,

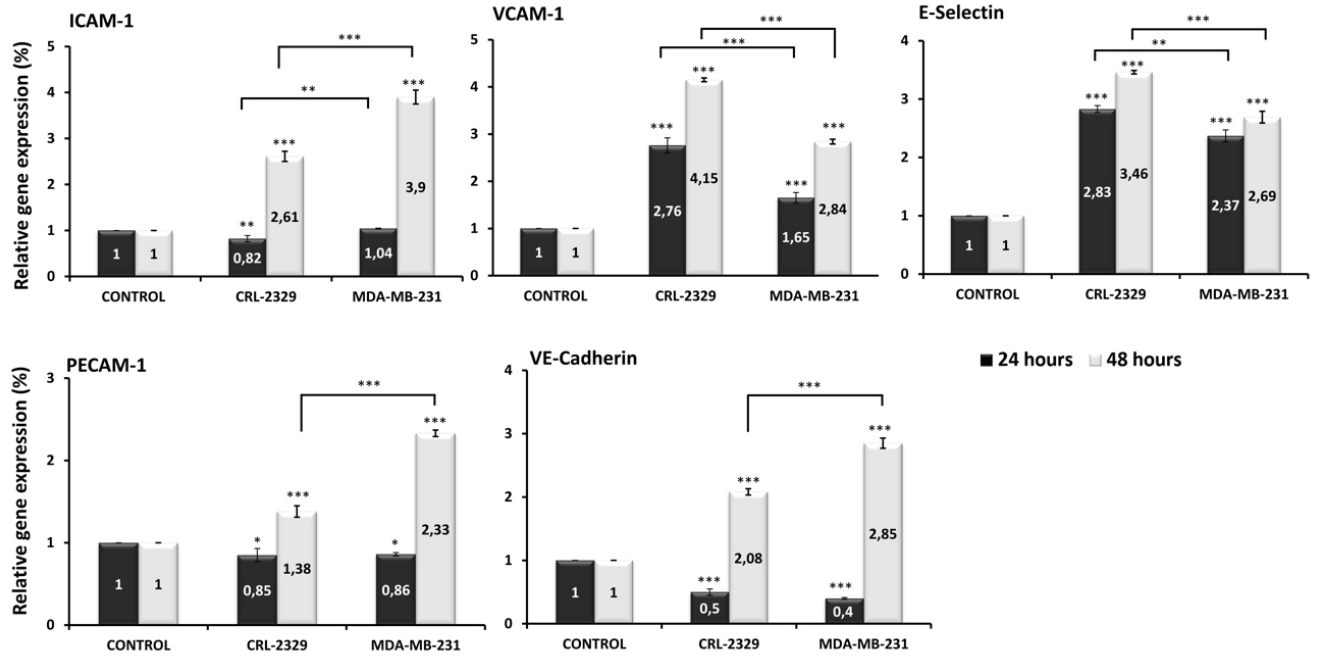

Figure 4. The relative mRNA expressions of *VE-cadherin, VCAM-1, ICAM-1, PECAM-1* and *E-Selectin* genes in ECs treated with CRL-4010-CM (Control), CRL-2329-CM and MDA-MB-231-CM. After detecting the Ct (cycle threshold) values of the reference (house-keeping) gene (GAPDH) and adhesion molecule genes, relative comparisons of expression levels were evaluated with the formula $2^{-\Delta\Delta CT}$. Data were presented as the mean ± standard deviation of three different experiments and shown as error bars. Asterisks above the bars show significance between each CM group and corresponding control group. *** indicates $p<0.001$. Other significances for comparisons are $p=0.0093$ for C *vs.* CRL-2329-CM and $p=0.0021$ for CRL-2329-CM vs. MDA-MB-231-CM in 24 h groups (ICAM-1); $p=0.003$ for CRL-2329-CM *vs.* MDA-MB-231-CM in 24 h groups (E-selectin); $p=0.0439$ for C *vs.* CRL-2329-CM and $p=0.0179$ for C *vs.* MDA-MB-231-CM in 24 h groups (PECAM-1).

ECs: Endothelial cells, ICAM-1: Intercellular adhesion molecule-1, VCAM-1: Vascular cell adhesion molecule-1, PECAM-1: Platelet endothelial cell adhesion molecule-1

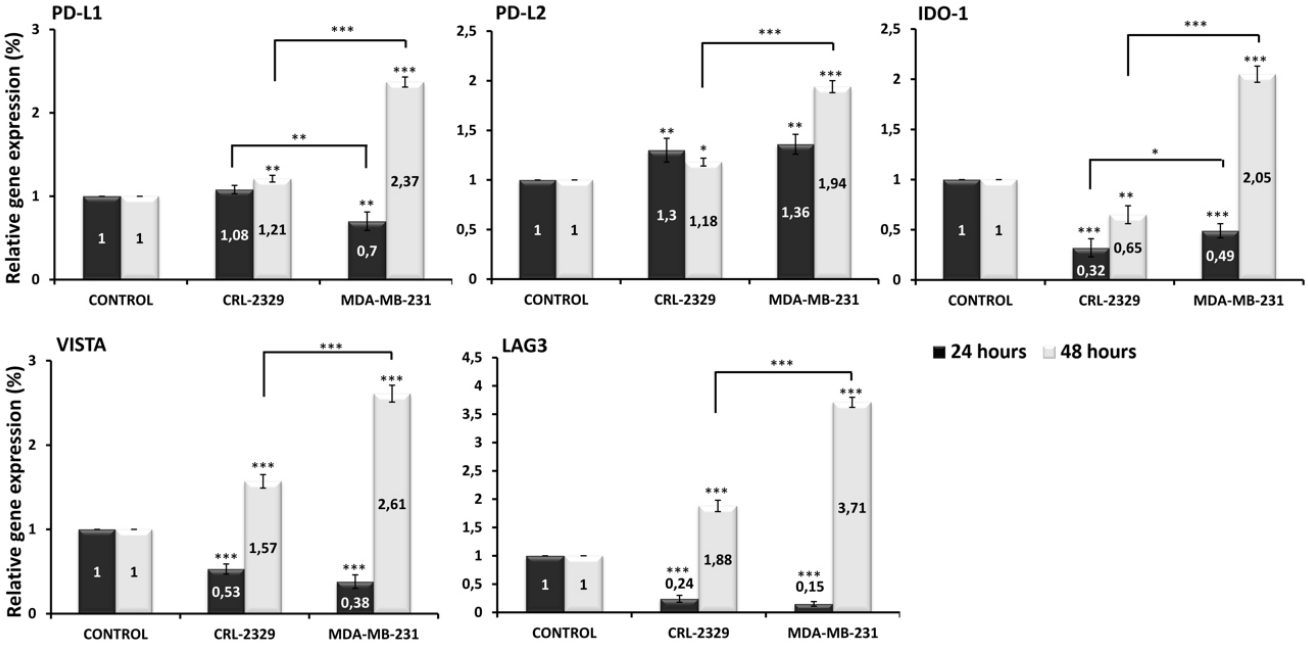

Figure 5. The relative mRNA expressions of PD-L1, VISTA, LAG3, PD-L2 ligands and IDO-1 enzyme in ECs treated with CRL-4010-CM (Control), CRL-2329-CM and MDA-MB-231-CM. After detecting the Ct (cycle threshold) values of the reference (house-keeping) gene (GAPDH) and the immunocheckpoints, relative comparisons of expression levels were evaluated with the formula $2^{-\Delta\Delta CT}$. Data were presented as the mean ± standard deviation of three different experiments and shown as error bars. Asterisks above the bars show significance between each CM group and corresponding control group. *** indicates p<0.001. Other significances for comparisons are p=0.0086 for C *vs.* MDA-MB-231-CM, p=0.0036 for CRL-2329-CM *vs.* MDA-MB-231-CM in 24 h groups and p=0.0028 for C *vs.* CRL-2329-CM in 24 h groups (PD-L1); p=0.0084 for C *vs.* CRL-2329-CM, p=0.0045 for C *vs.* MDA-MB-231-CM in 24 h groups, and p=0.0216 for C *vs.* CRL-2329-CM in 48 h groups (PD-L2); p=0.0354 for CRL-2329-CM *vs.* MDA-MB-231-CM in 24 h groups and p=0.0052 for C *vs.* CRL-2329-CM in 48 h groups (IDO-1).

ECs: Endothelial cells, IDO: Indoleamine 2,3-dioxygenase

respectively) (p<0.001, Figure 5). Additionally, only a slight increment in the expression of these genes was found in cells treated with CRL-2329-CM for 48 hours, except for the IDO-1 enzyme whose expression was significantly decreased (1.21-fold, p=0.0028; 1.18-fold, p=0.0216; 0.65-fold, p=0.0052; 1.57-fold, p<0.001; 1.88-fold, p<0.001, respectively) when compared to control (Figure 5). Cells in almost all 24-hour groups showed a significant decrease (p<0.001 for IDO-1, VISTA and LAG3) in these mRNA expressions except for PD-L2 whose expression mildly increased (p=0.0084 and p=0.0045) (Figure 5). It should also be noted that we did not observe any considerable result for the expression of *CTLA-4* and *IDO-2* genes in ECs. A signal was received only after the 37-38th cycles for *IDO-2* gene and no signal was detected in the 40-cycle of PCR reaction for *CTLA-4* gene. Therefore, we concluded that there were no expressions of these genes.

One of the striking results in immune checkpoint gene expression levels was that the increases were greater in EC cells treated with MDA-MB-231-CM.

Discussion

It is known that communication between cells is provided by various soluble factors released from cells. Moreover, it is known that conditioned media collected from cell lines are rich in secreted proteins including metalloproteases, growth factors, cytokines, and other soluble mediators, which contribute to cell survival, growth, invasion and differentiation of the cells with which they communicate (19). One of the cellular equipment in tumor microenvironmet is ECs which play critical roles in adhesion, intravasation and extravasation of tumor cells (8). In this process, ECs exhibit some functions as both having static structural properties and as active stromal regulatory cells (8,20).

While normal ECs remain in a quiescent state, the adhesion capacity of ECs can increase dramatically during cancer cell spread, and also the proliferation rate of tumor-associated ECs increases significantly. Adhesion receptors also undergo functional changes (8). In this respect, CM reflects the tumor microenvironment *in vitro*. CM secreted

from cancer cells has been used as a model to see how it affects molecules in the tumor microenvironment. Normal breast epithelial cells treated with CM from aggressive breast carcinoma cells were reported to increase cell proliferation and migration (21). However, breast cancer CM derived from MDA-MB-231 was shown to slightly reduce cell proliferation in ECs when applied for 72 hours (22). Consistent with this study, we found that MDA-MB-231-CM significantly decreased EC proliferation in the 24-hour group, but slightly decreased it in the 48-hour group. However, our study also revealed that while CRL-2329-CM did not affect cell proliferation in the 24-hour group, its effect increased EC proliferation in the 48-hour group. Consistent with previous work (21), this study showed that both CMs significantly increased the rate of cell migration.

ECs have important critical roles in supporting and promoting the growth and metastasis of cancer cells in tumor microenvironment (2,6). Because of these dramatic effects of ECs, we also investigated the effects of breast cancer CMs on the gene expression of adhesion molecules involved in the metastasis, and immune checkpoints which could be effective in immunosuppression and might indirectly support cancer growth.

ICAM-1, one of the immunoglobulin-like cell adhesion molecules, is expressed by various cell types such as ECs (23). It provides tight adhesion of leukocytes to the endothelium (23). Studies on ICAM-1 molecule have suggested that its increased expression in cancerous tissues mediates cell adhesion and metastasis (24,25). In a previous study, Western blotting of HUVEC extracts showed that ZR75.30 breast cancer-CM, but not MCF-7-CM, increased the levels of the adhesion proteins VCAM-1, E-selectin and ICAM-1 (8). Additionally, CM derived from ZR75.30 ductal carcinoma cells significantly increased the mRNA expression of these genes, but the researchers did not report such an effect for MCF-7-CM (8). Consistent with these studies, we found that ICAM-1 expression was significantly increased in ECs treated with both CRL-2329-CM and MDA-MB-231-CM for 48 hours compared to control. VCAM-1 adhesion molecule is a key regulator for leukocyte adhesion and transendothelial migration (26). It has been shown in some studies that there is a correlation between the high endothelial VCAM-1 expression and the metastasis of cancer cells to the other tissues (27,28). E-selectin, another important cell surface adhesion molecule, is expressed by activated ECs. Studies on E-selectin have shown that it has a direct effect on inducing angiogenesis, which is important for tumor growth and metastasis (29-31). We also showed, in the present study, the mRNA levels of E-selectin and VCAM-1 molecules were markedly increased in all groups as compared to the control group. Another adhesion molecule secreted by ECs, which is important for maintaining endothelial integrity, is PECAM-1 (32). PECAM-1 is implicated in a number of cellular processes, including extracirculatory leukocyte trafficking, angiogenesis and vascular permeability, supporting the growth and spread of tumor cells (33-35). In our study, while the expression levels of the *PECAM-1* gene decreased slightly in the 24-hour groups, it was found to be significantly increased in the 48-hour CRL-2329-CM and MDA-MB-231-CM treated groups. Similarly, we observed that VE-cadherin levels were significantly increased in ECs treated with both CMs in the 48-hour groups, but not in the 24-hour groups, where its mRNA levels were considerably diminished. VE-cadherin, which is present at low levels in normal ECs, was found to be expressed at higher levels in tumor-associated ECs. VE-cadherin increases more as the disease progresses, suggesting a role in angiogenesis rather than vasculogenesis (36,37). It should be noted that we could not find any result for the E-cadherin gene in ECs treated with CRL-2329-CM and MDA-MB-231-CM.

With regard to the immune checkpoint genes that are involved in the process of immune suppression, we have had obtained some striking results. PD-L1 and PD-L2, which are the members of the B7 family, are well known to bind to the corresponding inhibitory receptors on the surface of T-cells. These ligands are essential for the regulation of immune responses and the maintenance of immune tolerance (13). PD-L1 mRNA levels have been reported to be elevated in a number of tumor cell lines and are associated with high levels of immunosuppression (11). We found that the PD-L1 gene expression was detected in both 48-hour CM groups, and that its expression was particularly elevated in the MDA-MB-231-CM treated group compared to the control group. Similar results were also obtained for PD-L2 expression, but for this gene, we also detected in the 24-hour groups. Based on these data, it can be said that increased expressions of endothelial PD-L1 and PD-L2 may contribute to the establishment of a favorable immunosuppressive environment for tumor growth.

In this study, we also examined other important factors involved in immunosuppression. These were the enzymes IDO-1 and IDO-2, which metabolize tryptophan to kynurenine and play a role in tumor immune evasion and carcinogenesis (38). When the 48-hour groups of our study were evaluated, a significant increase in *IDO-1* gene expression levels was observed in ECs grown under the MDA-MB-231-CM conditions. In the other experimental conditions, we found decreases in *IDO-1* gene expressions (Figure 5). In addition, we could not obtain any result for *IDO-2* gene expression in ECs. Furthermore, we were unable to obtain any results for another immunomodulatory factor, CTLA-4.

VISTA is both a receptor for T-cells and a ligand for antigen presenting cells. This immune checkpoint gene has been suggested to suppress T-cell activation (17,18). High mRNA expression of VISTA was observed in some malignant tumor types (39-41). The other factor we have studied is LAG3, which displayed suppressive regulatory effects on T-cell responses and implicates T-cell dysfunction in the tumor microenvironment. LAG3 is highly expressed in tumor-associated lymph nodes, according to a dramatic study (42). Also, regulatory T-cells enhanced the expression of LAG3 ligand in PBMC and tumor sites isolated from patients with advanced (stage III and IV) cancer (42). In the present study, we also found similar results for these two genes. *VISTA* and *LAG3* genes were shown to significantly increase in the 48-hour experimental groups of CRL-2329-CM and MDA-MB-231-CM-treated ECs, but not in the 24-hour experimental groups.

In fact, we did not expect any results in ECs for the immune checkpoint molecules LAG3 and CTLA-4. We considered this as a sort of dysfunctional negative control. However, given the receptor function of the *LAG3* immunomodulatory molecule, it is dramatic that the LAG3 gene is expressed in ECs. As the functions of LAG3 are complex, further studies on LAG3 in particular are needed to understand the function of its expression in ECs.

Regarding the comparison of breast cancer cell types studied in the present study, we can say that the effects of the secretome obtained from MDA-MB-231 cells on the related genes are more aggressive. MDA-MB-231 is a breast cancer cell line with estrogen receptor negative (ER-), progesterone receptor negative (PR-) and human epidermal growth factor receptor negative (HER2-). Therefore, these cells are subgrouped as triple negative breast cancer cell type (TNBC) and associated with tumor-invasive and aggressive genes (43-45). Another cell type, CRL-2329, is a breast cancer cell line with ER+, PR+, HER2- characteristics, and classified as Luminal A type exhibiting relatively low aggressiveness (44). TNBC is considered to be more aggressive and causes a worse prognosis compared to other breast cancer subtypes. For example, TNBC exhibits increased PD-L1 expression. In a study, to evaluate the PD-L1 expression status *in vitro*, the researchers have compared the expression levels of PD-L1 in the human breast cancer cell lines and have reported that *PD-L1* gene is expressed relatively higher in MDA-MB-231 cells than that in CRL-2329 cells (HCC1500) (46). In another study, the possible immunomodulatory effects of MDA-MB-231 and BT-474 (Luminal B type) breast cancer cells induced by conditioned medium from bone marrow-derived mesenchymal stromal cells were examined *in vitro* and it was suggested that the expression levels of IDO, transforming growth factor-β and IL-10 were much higher in MDA-MB cells and these cell type had a strong immunosuppressive effects (47).

Consistent with the literature, the expression levels of the immune checkpoint genes we investigated were also found to be higher in ECs treated with MDA-MB-231 cell secretome. In addition, we showed that the expression levels of some adhesion molecules, especially ICAM-1, PECAM-1 and VE-cadherin, increased more in the MDA-MB-231 group, indicating more aggressive phenotype.

Conclusion

In conclusion, when the data we obtained are considered together, it emphasizes that ECs have essential roles in the interaction with cancer cells and immune system cells. This study has the potential to support the literature in this regard, but further studies are needed.

Ethics

Ethics Committee Approval: Our study was conducted on cell line and ethics committee approval is not required.

Informed Consent: Not necessary.

Authorship Contributions

Concept: M.Ş., Design: İ.T., E.Ş., M.Ş., Data Collection or Processing: İ.T., E.Ş., Analysis or Interpretation: İ.T., E.Ş., M.Ş., Literature Search: İ.T., E.Ş., M.Ş., Writing: M.Ş.

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References

1. Fahad Ullah M. Breast cancer: current perspectives on the disease status. Adv Exp Med Biol. 2019;1152:51-64.
2. Sahin M, Sahin E, Gumuslu S, Erdogan A, Gultekin M. DNA methylation or histone modification status in metastasis and angiogenesis-related genes: a new hypothesis on usage of DNMT inhibitors and S-adenosylmethionine for genome stability. Cancer Metastasis Rev. 2010;29:655-76.
3. Baghban R, Roshangar L, Jahanban-Esfahlan R, Seidi K, Ebrahimi-Kalan A, Jaymand M, et al. Tumor microenvironment complexity and therapeutic implications at a glance. Cell Commun Signal. 2020;18:59.
4. Sahin M, Sahin E, Koksoy S. Regulatory T cells in cancer: an overview and perspectives on cyclooxygenase-2 and Foxp3 DNA methylation. Hum Immunol. 2013;74:1061-8.
5. Sahin E, Sahin M. Epigenetical targeting of the FOXP3 gene by S-adenosylmethionine diminishes the suppressive capacity of regulatory T cells ex vivo and alters the expression profiles. J Immunother. 2019;42:11-22.

6. Sahin M, Sahin E, Gumuslu S. Cyclooxygenase-2 in cancer and angiogenesis. Angiology. 2009;60:242-53.

7. Lim WC, Olding M, Healy E, Millar TM. Human endothelial cells modulate CD4(+) T cell populations and enhance regulatory T cell suppressive capacity. Front Immunol. 2018;9:565.

8. Mejia-Rangel J, Cordova E, Orozco L, Ventura-Gallegos JL, Mitre-Aguilar I, Escalona-Guzman A, et al. Pro-adhesive phenotype of normal endothelial cells responding to metastatic breast cancer cell conditioned medium is linked to NFkappaB-mediated transcriptomic regulation. Int J Oncol. 2016;49:2173-85.

9. Oppenheimer SB. Cellular basis of cancer metastasis: A review of fundamentals and new advances. Acta Histochem. 2006;108:327-34.

10. Park R, Winnicki M, Liu E, Chu WM. Immune checkpoints and cancer in the immunogenomics era. Brief Funct Genomics. 2019;18:133-9.

11. Zheng Y, Fang YC, Li J. PD-L1 expression levels on tumor cells affect their immunosuppressive activity. Oncol Lett. 2019;18:5399-407.

12. Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, et al. PD-L2 Expression in human tumors: relevance to anti-PD-1 therapy in cancer. Clin Cancer Res. 2017;23:3158-67.

13. Rodig N, Ryan T, Allen JA, Pang H, Grabie N, Chernova T, et al. Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. Eur J Immunol. 2003;33:3117-26.

14. Wei L, Zhu S, Li M, Li F, Wei F, Liu J, et al. High ındoleamine 2,3-dioxygenase ıs correlated with microvessel density and worse prognosis in breast cancer. Front Immunol. 2018;9:724.

15. Solinas C, Migliori E, De Silva P, Willard-Gallo K. LAG3: the biological processes that motivate targeting this immune checkpoint molecule in human cancer. Cancers (Basel). 2019;11

16. Syn NL, Teng MWL, Mok TSK, Soo RA. De-novo and acquired resistance to immune checkpoint targeting. Lancet Oncol. 2017;18:e731-e41.

17. Le Mercier I, Chen W, Lines JL, Day M, Li J, Sergent P, et al. VISTA regulates the development of protective antitumor immunity. Cancer Res. 2014;74:1933-44.

18. Boger C, Behrens HM, Kruger S, Rocken C. The novel negative checkpoint regulator VISTA is expressed in gastric carcinoma and associated with PD-L1/PD-1: A future perspective for a combined gastric cancer therapy? Oncoimmunology. 2017;6:e1293215.

19. Abbineni PS, Tang VT, da Veiga Leprevost F, Basrur V, Xiang J, Nesvizhskii AI, et al. Identification of secreted proteins by comparison of protein abundance in conditioned media and cell lysates. Anal Biochem. 2022;655:114846.

20. Franses JW, Baker AB, Chitalia VC, Edelman ER. Stromal endothelial cells directly influence cancer progression. Sci Transl Med. 2011;3:66ra5.

21. Guo J, Liu C, Zhou X, Xu X, Deng L, Li X, et al. Conditioned medium from malignant breast cancer cells induces an EMT-like phenotype and an altered N-glycan profile in normal epithelial MCF10A cells. Int J Mol Sci. 2017;18

22. Gomez EO, Chirino YI, Delgado-Buenrostro NL, Lopez-Saavedra A, Meraz-Cruz N, Lopez-Marure R. Secretome derived from breast tumor cell lines alters the morphology of human umbilical vein endothelial cells. Mol Membr Biol. 2016;33:29-37.

23. Lawson C, Ainsworth M, Yacoub M, Rose M. Ligation of ICAM-1 on endothelial cells leads to expression of VCAM-1 via a nuclear factor-kappaB-independent mechanism. J Immunol. 1999;162:2990-6.

24. Zhang P, Goodrich C, Fu C, Dong C. Melanoma upregulates ICAM-1 expression on endothelial cells through engagement of tumor CD44 with endothelial E-selectin and activation of a PKCalpha-p38-SP-1 pathway. FASEB J. 2014;28:4591-609.

25. Rosette C, Roth RB, Oeth P, Braun A, Kammerer S, Ekblom J, et al. Role of ICAM1 in invasion of human breast cancer cells. Carcinogenesis. 2005;26:943-50.

26. Kong DH, Kim YK, Kim MR, Jang JH, Lee S. Emerging roles of vascular cell adhesion molecule-1 (VCAM-1) in immunological disorders and cancer. Int J Mol Sci. 2018;19:1057.

27. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. Nature. 2005;436:518-24.

28. Siyasi M, Mahjoubi F, Mahjoubi B, Shabani S, Shen N. Study of VCAM-1 gene expression in normal and tumoral tissues in patients with colorectal cancer. Journal of Biotechnology and Biomedical Science. 2017;1:19-26.

29. Hebbar M, Peyrat JP. Significance of soluble endothelial molecule E-selectin in patients with breast cancer. Int J Biol Markers. 2000;15:15-21.

30. McCarthy JB, Skubitz AP, Iida J, Mooradian DL, Wilke MS, Furcht LT. Tumor cell adhesive mechanisms and their relationship to metastasis. Semin Cancer Biol. 1991;2:155-67.

31. Koch AE, Halloran MM, Haskell CJ, Shah MR, Polverini PJ. Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. Nature. 1995;376:517-9.

32. Abraham V, Cao G, Parambath A, Lawal F, Handumrongkul C, Debs R, et al. Involvement of TIMP-1 in PECAM-1-mediated tumor dissemination. Int J Oncol. 2018;53:488-502.

33. Muller WA. The role of PECAM-1 (CD31) in leukocyte emigration: studies in vitro and in vivo. J Leukoc Biol. 1995;57:523-8.

34. Newman PJ, Newman DK. Signal transduction pathways mediated by PECAM-1: new roles for an old molecule in platelet and vascular cell biology. Arterioscler Thromb Vasc Biol. 2003;23:953-64.

35. Kashani-Sabet M, Liu Y, Fong S, Desprez PY, Liu S, Tu G, et al. Identification of gene function and functional pathways by systemic plasmid-based ribozyme targeting in adult mice. Proc Natl Acad Sci U S A. 2002;99:3878-83.

36. Carter WB, Niu G, Ward MD, Small G, Hahn JE, Muffly BJ. Mechanisms of HER2-induced endothelial cell retraction. Ann Surg Oncol. 2007;14:2971-8.

37. Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, et al. Alterations in vascular gene expression in invasive breast carcinoma. Cancer Res. 2004;64:7857-66.

38. Bilir C, Sarisozen C. Indoleamine 2,3-dioxygenase (IDO): Only an enzyme or a checkpoint controller? J Oncol Sci. 2017;3:52-6.

39. Huang X, Zhang X, Li E, Zhang G, Wang X, Tang T, et al. VISTA: an immune regulatory protein checking tumor and immune cells in cancer immunotherapy. J Hematol Oncol. 2020;13:83.

40. Mulati K, Hamanishi J, Matsumura N, Chamoto K, Mise N, Abiko K, et al. VISTA expressed in tumour cells regulates T cell function. Br J Cancer. 2019;120:115-27.

41. Rosenbaum SR, Knecht M, Mollaee M, Zhong Z, Erkes DA, McCue PA, et al. FOXD3 regulates VISTA expression in melanoma. Cell Rep. 2020;30:510-24.e6.

42. Camisaschi C, Casati C, Rini F, Perego M, De Filippo A, Triebel F, et al. LAG-3 expression defines a subset of CD4(+)CD25(high) Foxp3(+) regulatory T cells that are expanded at tumor sites. J Immunol. 2010;184:6545-51.

43. Dai X, Cheng H, Bai Z, Li J. Breast cancer cell line classification and its relevance with breast tumor subtyping. J Cancer. 2017;8:3131-41.

44. Witt BL, Tollefsbol TO. Molecular, cellular, and technical aspects of breast cancer cell lines as a foundational tool in cancer research. Life (Basel). 2023;13:2311.

45. Aysola K, Desai A, Welch C, Xu J, Qin Y, Reddy V, et al. Triple negative breast cancer - an overview. Hereditary Genet. 2013;2013.

46. Zhou Y, Yamamoto Y, Takeshita F, Yamamoto T, Xiao Z, Ochiya T. Delivery of miR-424-5p via extracellular vesicles promotes the apoptosis of MDA-MB-231 TNBC cells in the tumor microenvironment. Int J Mol Sci. 2021;22:844.

47. Arenas-Luna VM, Montesinos JJ, Cortes-Morales VA, Navarro-Betancourt JR, Peralta-Ildefonso J, Cisneros B, et al. In vitro evidence of differential immunoregulatory response between MDA-MB-231 and BT-474 breast cancer cells induced by bone marrow-derived mesenchymal stromal cells conditioned medium. Curr Issues Mol Biol. 2022;45:268-85.