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Original Article

Effect of soluble factors derived from ZR 75.30 breast cancer cells on endothelial activation

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Abstract: In this study, we analyzed soluble factors secreted by two Estrogen Receptor Positive (ER-α) human breast cancer cell lines, ZR 75.30 (luminal B) and MCF7 (luminal A), and evaluated their effect on endothelial activation. The composition of tumoral soluble factors (TSFs) was analyzed by ELISA (Bio-Plex). TSFs from ZR 75.30 cells expressed higher levels of TNF, IFN-γ, IL-6, and IL-8 compared to TSFs from MCF-7 cells. TSFs from ZR 75.30 cells induced a pro-adhesive phenotype in human umbilical vein endothelial cells (HUVECs), as characterized by increased monocytic cell adhesion, adhesion molecule expression and NF-κB activation and decreased IκB-α expression. Conversely, TSFs from MCF-7 cells exerted none of these effects on HUVECs. We then added TNF, IFN-γ, IL-6 or IL-8 alone or in combination with TSFs from MCF-7 cells to HUVECs. Only the combinations that included TNF induced endothelial activation. A neutralizing antibody against IL-1β (this cytokine was not measured in the ELISA) had a modest blocking effect on cellular adhesion or the expression of adhesion molecules induced by TSFs from ZR 75.30 cells in HUVECs. However neutralizing antibodies against TNF, IFN-γ, IL-6 or IL-8 had no effect. Our results suggest that although TNF is an inducer of endothelial cell activation, it is not the only molecule that is responsible for this effect in TSFs from ZR 75.30 cells.

Keywords: Tumoral soluble factors, TNF, endothelial activation, breast cancer, endothelial cell adhesion molecules

Introduction

Breast cancer is the most commonly occurring malignancy in women and is responsible for approximately 522,000 deaths annually worldwide [http://gco.iarc.fr/today], and most of these deaths are associated with metastasis to the lung, bone, brain or liver. Metastasis is a complex process involving multiple steps, including i) invasion across the basement membrane, ii) intravasation into the vascular or lymphatic system, iii) survival in the bloodstream, iv) binding to the wall of blood vessels, v) extravasation, vi) aggressive colonization and vii) growth in the target organ [1]. Tumor cells secrete a complex mixture enriched in cytokines, chemokines, growth factors, and enzyme modulators that contribute to the tumor microenvironment. Consequently, the intrinsic properties of tumor cell secretion products are determinants of the risk and organ specificity of metastases [2]. Recent studies have suggested that the recruitment of normal cells from target organs contributes to intravasation and colonization during metastasis. Indeed, endothelial cells from the target organ are the first normal cellular components that appear to collaborate with metastatic cells during extravasation [3].

Interaction between metastatic cells and the vascular endothelial wall appears to be a necessary step for metastatic organ invasion and likely requires adhesion, diapedesis and extravasation. Although the precise mechanisms that mediate this interaction remain poorly defined [4], such interactions between endothelial cells and other cell types require growth factors, chemokines and proinflammatory cytokines, such as VEGF, IL-8, IL-6 and TNF. Interestingly, these factors have been associated with metastasis in a variety of cancers [5, 6].

A previous work showed that tumor soluble factors (TSFs) from breast cancer cells (ZR 75.30) enhanced the adhesion of monocytic cells to human umbilical vein endothelial cells (HUVECs) and NF-κB activation, while TSFs from MCF-7 cells did not. Additionally it was shown that cytokines such as TNF, IL-1β, IL-6 and IFN-γ and chemokines like IL-8 are more abundant in the former than in the latter cell line [7]. However, it was not evaluated if these components are responsible for endothelial activation. In this work, we hypothesized that if HUVECs are exposed to TSFs from MCF-7 cells supplemented with the concentrations of cytokines secreted by ZR 75.30 cells (TNF, IFN-γ, IL-6 or IL-8), activation of HUVECs will be observed. Also, in HUVECs exposed to TSFs from ZR 75.30 plus neutralizing antibodies against all these cytokines, activation will be prevented. To test this, HUVECs were exposed to TSFs derived from MCF-7 and ZR 75.30 cells, and the acquisition of an activated endothelial state was evaluated. The results revealed that TSFs from ZR 75.30 cells induced cellular and molecular changes that were consistent with an endothelial activation phenotype, including the increased adhesion of monocytes U937, expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and activation of nuclear factor κB (NF-κB). Of the four cytokines present at high concentrations in TSFs from ZR 75.30 cells, only recombinant TNF induced endothelial activation. However, the depletion of TNF from TSFs derived from ZR 75.30 cells did not reduce endothelial cell activation, suggesting that additional factors contribute to the endothelial activation phenotype.

Materials and methods

Generation of TSF

MCF-7 cells (low metastatic potential) and ZR 75.30 (high metastatic potential) were used. To obtain TSF, conditioned media derived from these cells were collected as previously described [7, 8], and the samples were analyzed by Bio-Plex ELISA (Bio-Rad) for 17 cytokines or chemokines (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, TNF, IFN-γ, GM-CSF, G-CSF, MCP-1, MIP-1b, Eotaxin-1, FGF, IP-10, MIP-1a, PDGF, RANTES and VEGF). The concentrated preparation containing the TSFs was stored at 4°C until further use.

Collection of HUVECs and cell culture

HUVECs were obtained from the umbilical cords of healthy women, who had undergone uncomplicated term pregnancies according to a method described previously [9]. Umbilical cords were obtained from the General Hospital "Dr. Manuel Gea González", with approval from the Ethics and Research Committee of the hospital and the informed consent of the donors (approval registration number: 11-17-2017). The myeloma cell line (U937) and the breast cancer cell lines (ZR 75.30 and MCF-7) were obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (Gibco, BRL, Grand Island NY). All cultures were used under endotoxin-free conditions.

Endothelial activation assays

Endothelial activation was evaluated in response to the positive control TNF (10 ng/ml), TSFs (5 μg/ml of different TSFs), and recombinant cytokines (R&D Systems, Minneapolis, MN) at concentrations found in TSFs, as indicated in the corresponding figure legends. For such purpose, a cellular adhesion assay, expression of adhesion molecules and activation of the NF-κB pathway were performed as previously described [7]. To avoid the presence of LPS, the TSFs were treated with polymyxin-D (10 μg/ml); 10 ng/ml LPS was also added as a control.

Cellular adhesion assay

HUVECs were treated with the stimuli described above for 3 hours. Then they were co-cultured for another 3 hours with naive U-937 cells (radiolabeled 48 hours before with 1 μCi/ml [^{3}H]-thymidine (NEN, Boston, MA)). Unattached U-937 were washed away and the rest of the cells were lysed with 0.2 N NaOH. Radioactivity

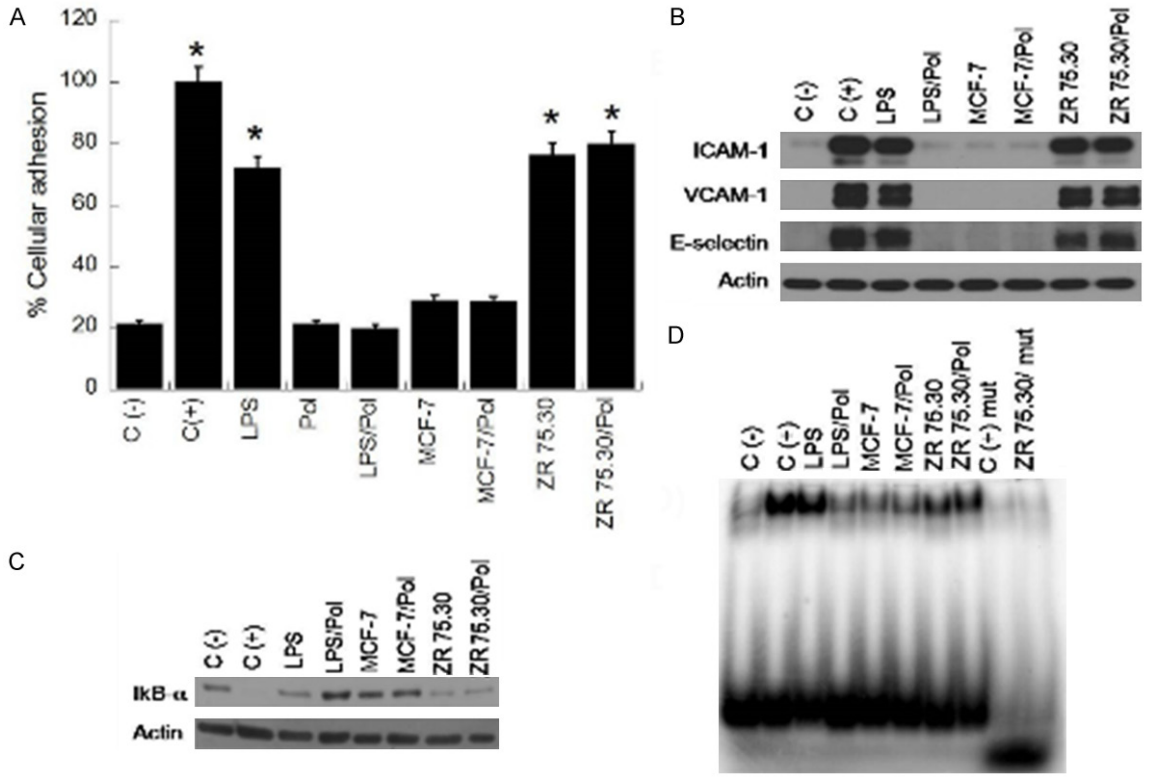

Figure 1. Endothelial activation induced by TSFs from ZR 75.30 and MCF-7 cells. A: Induction of pro-adhesive phenotype in HUVECs by TSFs derived from breast cancer cells (MCF-7 or ZR 75.30) after 3 h of treatment, followed by 3 h of co-incubation with U937 cells. B: Western blot of adhesion molecules after 6 h of treatment. C: Western blot of IκB-α after 20 min of treatment. D: Activation of NF-κB in nuclear protein extracts from HUVECs treated for 20 min was analyzed by EMSA. HUVECs were treated with TNF (10 ng/ml) (C [+]), LPS (10 ng/ml), polymyxin-D (10 μg/ml) or TSFs from MCF-7 or ZR 75.30 cells (5 μg/ml). C (-): untreated cells.

level was determined using a scintillation counter (Beckman LS6000SC, St Louis, MO); the radioactivity is directly proportional to the number of U937 cells attached to the HUVECs.

Expression of adhesion molecules

ICAM-1, VCAM-1, and E-selectin protein level in HUVEC was measured after 6 hours of treatment with the stimuli described above. After lysing cells in RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0), total protein concentration was determined using a commercial Bradford reagent assay (Bio-Rad). Western blot assays were performed using 20 μg of total protein for the detection of VCAM-1 and E-selectin and 10 μg of total protein for the detection of ICAM-1 as described previously [10]. All primary antibodies were purchased from Santa Cruz, Santa Cruz, CA, and the secondary antibodies from Pierce, Rockford, IL.

Activation of NF-κB pathway

HUVEC cells were treated with the stimuli described above for 20 minutes. Cytoplasmic and nuclear protein extracts were obtained and an EMSA assay was performed as described previously [11]. Briefly 8 μg of nuclear protein extract was incubated with γ-ATP-[^{32}P] labeled oligonucleotides containing a decameric κB site (5'AGTTGAGGGGACTTTCCCAGGC) (Santa Cruz). The level of IκB-α protein expression, in 10 μg of cytoplasmic protein extracts, was analyzed by western blotting as described previously [10].

TSFs from ZR 75.30 cells depleted of TNF, IFN-γ, IL-6 or IL-8 or all these factors simultaneously

TSFs from ZR 75.30 cells were treated for 1 h with neutralizing antibodies against TNF, IFN-γ, IL-6 or IL-8 or of all factors simultaneously. The neutralizing antibodies used were sufficient to

Table 1. Concentration of 17 Tumor Secreted Factors of MCF-7 and ZR-75.30, determined using an ELISA Bio Plex system

Cytokines	MCF-7 (ng/ml)	ZR-75.30 (ng/ml)	Ratio MCF-7:ZR 75.30
TNF	0.14	1.4	1:10
IFN-γ	0.74	3.18	1:4
IP-10	0.45	1.05	1:2
IL-1ra	0.75	1.13	1:1
IL-4	0.06	0.07	1:1
IL-5	0.09	0.06	1:1
IL-6	0.16	2.99	1:18
IL-7	0.42	0.22	2:1
IL-9	0.33	0.36	1:1
IL-10	0.32	0.22	3:2
IL-12	0.23	0.25	1:1
IL-13	0.06	0.04	1:1
IL-17	0.42	0.49	1:1
Chemokines	MCF-7 (ng/ml)	ZR-75.30 (ng/ml)	Ratio MCF-7:ZR 75.30
IL-8	0.14	1.44	1:10
EOTAXIN	0.31	0.87	1:1
Growth Factors	MCF-7 (ng/ml)	ZR-75.30 (ng/ml)	Ratio MCF-7:ZR 75.30
G-CSF	0.13	0.40	1:3
FGF	0.14	0.26	1:1
PDGF	0.58	1.13	1:2

neutralize 10 ng/ml of each cytokine in the TSFs (anti-TNF: 1.4 ng/ml; anti-IFN-γ: 2.2 ng/ml; anti-IL-6: 3.0 ng/ml; and anti-IL-8: 1.4 ng/ml). All antibodies were purchased from R&D Systems (Minneapolis, MN).

Statistical analysis

The results in all graphs, are presented as, the mean of at least three independent experiments, performed using different primary culture HUVEC batches. The differences between variables were tested using the Student's t-test and, $P<0.01$ was considered to be statistically significant. Kaleida Graph software was used for the data analysis.

Results

TSF-induce endothelial activation

We prepared new batches of TSFs from MCF-7 and ZR 75.30 cells to analyze the relevance of TNF, IFN-γ, IL-6 or IL-8 in promoting endothelial activation as previously described [7].

The present batch of TSFs from ZR 75.30 cells increased cellular adhesion capacity of HUVEC 2.5-fold compared to the unstimulated cells; in contrast, TSFs from MCF-7 cells exerted no effect. To verify that the pro-adhesive phenotype induced by TSFs from ZR 75.30 cells did not occur due to potential contamination with LPS, the TSFs were treated with polymyxin-D, which did not affect the cellular adhesion induced by the TSFs, making the presence of LPS in the preparations unlikely (**Figure 1A**). In addition to cellular adhesion, other events are related to endothelial activation, such as the expression of adhesion molecules and the activation of NF-κB. Compared to untreated control, the TSFs from ZR 75.30 cells promoted the expression of ICAM-1, VCAM-1, and E-selectin (**Figure 1B**); in contrast, the TSFs from MCF-7 cells exerted no effect on the expression of these adhesion molecules. Furthermore, the TSFs from ZR 75.30 cells reduced the content of IκB-α (**Figure 1C**), and the activation of NF-κB was corroborated by an EMSA analysis (**Figure 1D**). Conversely, the TSFs from MCF-7 cells exerted no effect on either of these events.

TNF is the only product capable of endothelial activation and does not synergize with TSF from MCF-7 cells

Considering that different cytokines and chemokines can promote a pro-adhesive phenotype in HUVECs, we measured the expression of 17 such factors in the TSFs, using an ELISA Bio-Plex system (**Table 1**). The TSFs from ZR 75.30 cells displayed a higher level of TNF, IFN-γ, IL-6, IP-10, IL-8, eotaxin, and G-CSF than the TSFs from MCF-7 cells. The ratios (MCF-7:ZR 75.30) of these levels were as follows: TNF (1:10), IFN-γ (1:4), IL-6 (1:18), IP-10 (1:2), IL-8 (1:10), eotaxin (1:2), and G-CSF (1:3). Additionally it is worth noting that MCF-7 TSFs appear to contain more IL-7 and IL-10 than ZR 75.30 TSFs.

The ability of TSFs from ZR 75.30 cells to induce HUVECs activation may be due to the high con-

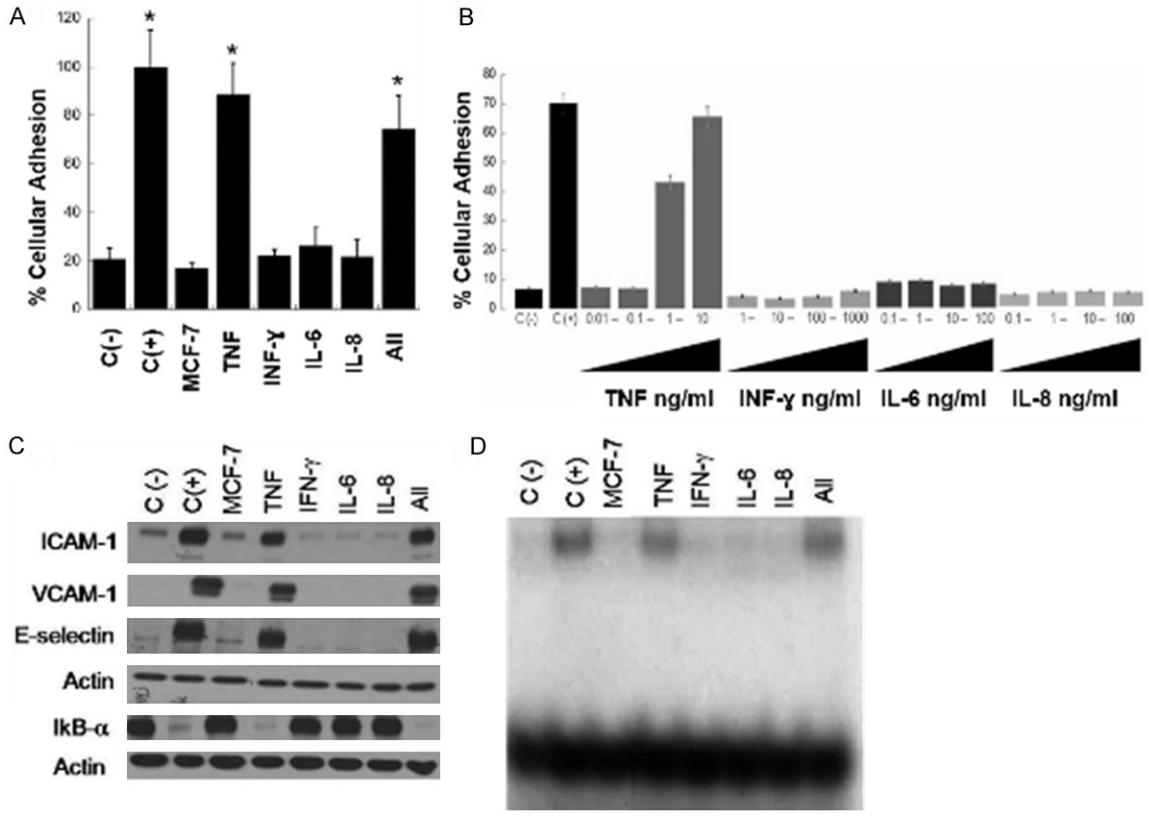

Figure 2. Endothelial activation induced by TNF, IFN-γ, IL-6 or IL-8. (A) Induction of pro-adhesive phenotype in HUVECs by recombinant cytokines alone or in combination (All), as in **Figure 1A**. (B) Same as (A) but with different concentrations of the recombinant cytokines, at logarithmic intervals. (C) Western blot of of adhesion molecules after 6 h of treatment (upper 4 lanes) and IκB-α after 20 min of treatment (lower 2 lanes. (D) Activation of NF-κB in nuclear protein extracts from HUVECs treated for 20 min was analyzed by EMSA. HUVECs were treated with TNF (1.3 ng/ml), IFN-γ (2.4 ng/ml), IL-6 (2.8 ng/ml), or IL-8 (1.2 ng/ml). Legends as in **Figure 1**.

centration of cytokines and chemokines, such as TNF, IFN-γ, IL-6 and IL-8, present in the samples. To determine whether any of these factors was responsible for the observed effects, we tested each factor on the activation of HUVECs individually or in combination using recombinant molecules. In all cases, these factors were added at the concentrations found in the TSFs from ZR 75.30 cells.

TNF alone or in combination with IFN-γ, IL-6 and IL-8 induced a 3.2- and 2.5-fold increase in cellular adhesion, respectively, compared to the control (**Figure 2A**). In order to provide more evidence that IFN-γ, IL-6 and IL-8 do not induce by themselves endothelial activation, we performed an adhesion assay evaluating different concentrations at logarithmic intervals of the recombinant cytokines (**Figure 2B**). Similar results were obtained for the expression of ICAM-1, VCAM-1, and E-selectin (**Figure 2C**, upper 4 lanes). The mechanism employed by TNF was dependant on NF-κB, as there was a reduction on the cytoplasmic content of IκB-α (**Figure 2C**, lower 2 lanes), and the nuclear extract of cells treated with TNF alone or in combination with the other cytokines induced a shift on the mobility of probe with a consensus NF-κB site (**Figure 2D**). Unexpectedly, treatment with IFN-γ, IL-6, or IL-8 alone exerted no effect on any of the events evaluated.

Considering that TSFs from MCF-7 cells did not have effect on endothelial activation, we hypothesized that when supplemented with individual cytokines or a combination of them, endothelial activation could be induced. Results were similar to those obtained in **Figure 2**. TNF alone or in combination with all the cytokines induced: an increase of: i) 2.5 and 2.0 fold in the cellular adhesion over control, respectively (**Figure 3A**), ii) an increased ex-

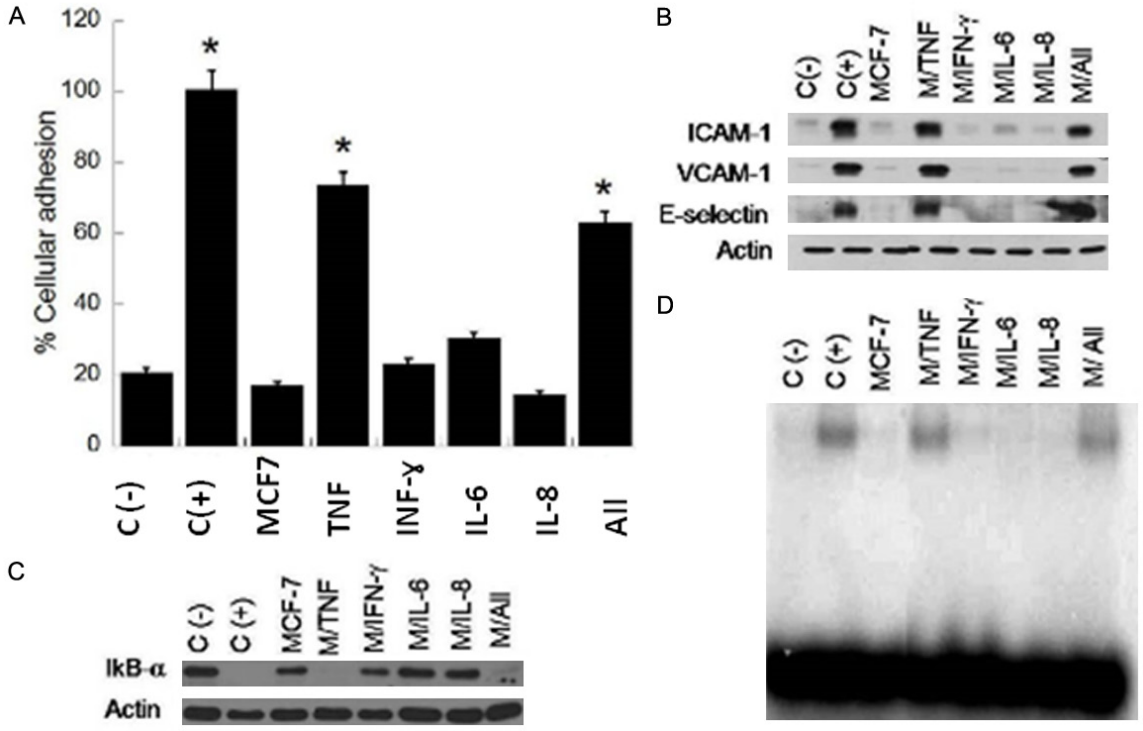

Figure 3. Endothelial activation by TSFs of MCF-7 supplemented with one or all of the following recombinant cytokines: TNF, IFN-γ, IL-6, or IL-8. A: Adhesion Assay, as in **Figure 1A**. B: Western blot of adhesion molecules after 6 h of treatment. C: Western blot of IκB-α after 20 min of treatment. D: Activation of NF-κB in nuclear protein extracts from HUVECs treated for 20 min was analyzed by EMSA. Legends as in **Figure 1**. Cytokine concentrations as in **Figure 2**.

pression of all adhesion molecules evaluated (**Figure 3B**), iii) reduced content of IκB-α (**Figure 3C**), and iv) activation of NF-κB (**Figure 3D**).

Taken together, these results suggest that TNF is at least partially responsible for the endothelial activation induced by TSFs from ZR 75.30 cells, and does not synergize with other factors present in TSFs from MCF-7 cells.

Effect of TSFs from ZR 75.30 cells depleted of TNF, IFN-γ, IL-6, IL-8 or IL-1β

To evaluate if there are specific TSF produced by ZR 75.30 cells that play a key role in the induction of endothelial activation, biological assays were performed using neutralizing antibodies against TNF, IFN-γ, IL-6, IL-8 or IL-1β. Interestingly, cell adhesion and expression of cell adhesion molecules were not affected when TSFs from ZR 75.30 cells were depleted of TNF. Similarly, neutralizing any of the others cytokines or chemokines had no effect on cell adhesion nor on expression of cell adhesion molecules (**Figure 4A**). A likely candidate for endothelial cell activation after TNF was removed is IL-1β. The addition of neutralizing antibodies against this cytokine resulted in a modest interference with the induction of the pro-adhesive phenotype (2%). Combined neutralization of TNF and IL-1β had a similar effect, and the combination of all five neutralizing antibodies reduced adhesion only 9% more.

Discussion

During metastasis cancer cells disseminate through the vascular system to distant organs and contribute to the high mortality associated to cancer [12]. The vascular microenvironment at the target organ of metastasis is recognized as an important contributor to this state of dissemination process and receives factors from the host organ and the metastatic cells [13]. The initial relevant cell-cell interactions that occur during this process include physical interactions and adhesion between metastatic cells and vascular and lymphatic endothelial cells [14]. A variety of molecular interactions occur between breast cancer cells and endothelial

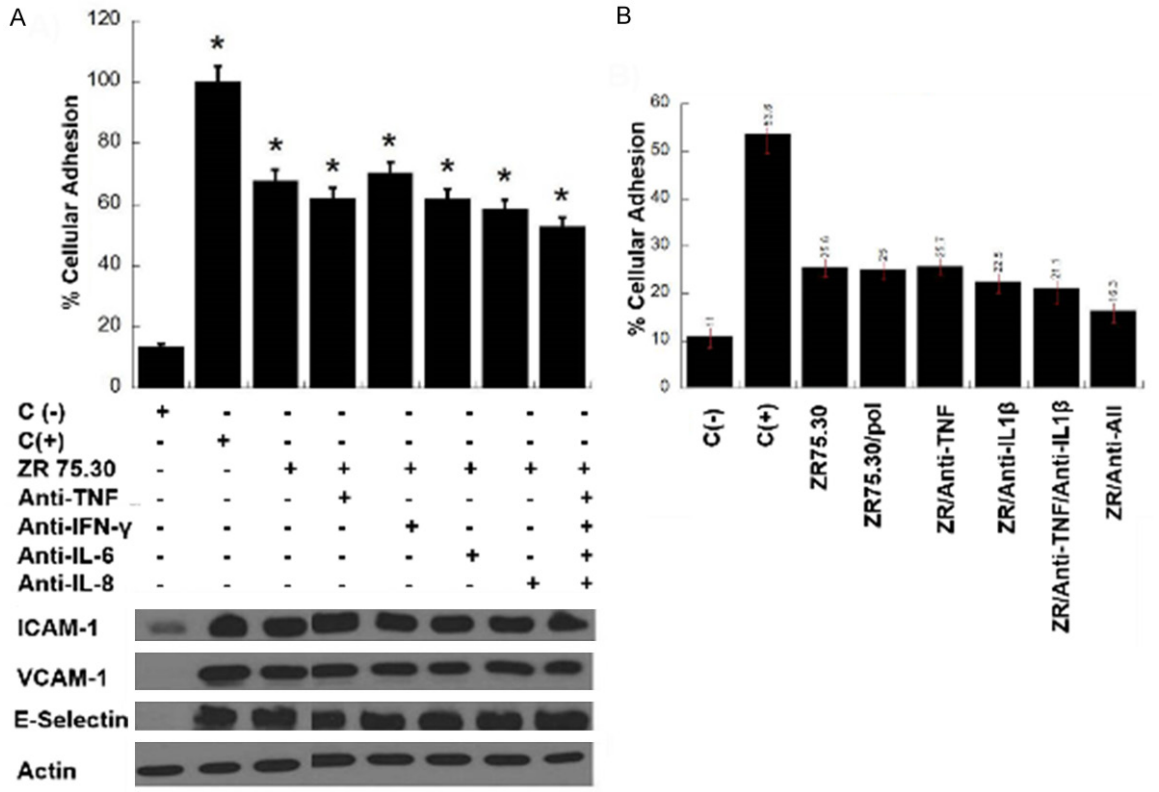

Figure 4. Endothelial activation by TSFs from ZR 75.30 treated with neutralizing antibodies against one or all of the following cytokines TNF, IFN-γ, IL-6, or IL-8. A: Upper graph: Induction of pro-adhesive phenotype in HUVECs by depleted TSFs from ZR 75.30 cells, using individually antibodies or their combination, as in **Figure 1A**. Lower part: Western blot of adhesion molecules after 6 h of treatment. HUVECs were treated with TSFs (5 μg/ml), and/or anti-TNF (1.4 ng/ml), anti-IFN-γ (2.2 ng/ml), anti-IL-6 (3.0 ng/ml), and/or anti-IL-8 (1.4 ng/ml) antibodies. B: Induction of pro-adhesive phenotype in HUVECs by depleted TSFs from ZR 75.30 cells, using neutralizing antibodies against IL-1β. Legends as in **Figure 1**, anti-TNF, anti-IFN-γ, anti-IL-6, anti-IL-8 and anti-IL-1β indicated addition of corresponding neutralizing antibodies.

cells suggesting that non transformed cells are recruited by cancer cells through cell-cell interactions as well as tumor derived soluble factors [15]. Normal, resident or recruited cell types, together with metastatic cells present at the site of metastasis, secrete enzymes, extracellular matrix, growth factors, cytokines, chemokines and other molecules that mediate cell-to-cell communication [16, 17]. We have previously described that TSFs from ZR 75.30 cells induced a pro-adhesive phenotype in HUVECs, whereas TSFs from MCF-7 cells did not [7], and we confirmed this with the new batch of TSFs used in this study (**Figure 1**).

This difference is associated with the more aggressive origin of ZR 75.30 cells that exhibit: a high invasive potential to bone [18], resistance to apoptosis [19], active migration and invasion [20], high proliferation rates, adherence to different substrates and invasive capacity [21]. In contrast, MCF-7 cells were derived from the pleural effusion from a woman with no metastasis, and are less aggressive compared to other breast cancer cell lines [22-26].

The analysis of the contents of TSFs from MCF-7 and ZR 75.30 cells revealed cytokines and chemokines related to inflammation and endothelial activation (TNF, IFN-γ, IP-10, IL-6, IL-8, and G-CSF), which may contribute to the pro-adhesive phenotype induced by the TSFs from ZR 75.30 cells (**Table 1**) [7]. Moreover, these cytokines and chemokines are produced by a variety of cancer cells to modulate tumor growth [27]. Interestingly, TNF, IFN-γ, IL-6 and IL-8 have also been associated with tumor cell dissemination in both murine experimental models and human metastases [27-31]. Despite their strong association with tumor progression, the precise mechanisms by which

these inflammation-related factors contribute to tumor cell dissemination, as well as the stage at which these factors participate in this process, are unknown. As endothelial cells express functional receptors for TNF [32], IFN-γ [33], IL-6 [34] and IL-8 [35], the resting endothelial phenotype can change to an activated state in which the cells participate in the adhesion and extravasation of metastatic cells. A central mediator for endothelial cell activation is NF-κB, both in response to inflammatory cytokines as well as TSFs [7, 36, 37].

Endothelial activation is characterized by the degradation of IκB-α in the cytoplasm, resulting in the release of the transcription factor NF-κB, which translocates to the nucleus activating transcription of adhesion molecules (ICAM-1, VCAM-1 and E-selectin), contributing to a pro-adhesive phenotype [38].

TNF, IFN-γ, IL-6 and IL-8 were found to be present at high concentrations in the TSFs from ZR 75.30 cells, and consequently we evaluated the individual effect of each of these cytokines when added at the same concentrations found in TSFs (**Figure 2**), as well as their combined effect in the presence of TSFs from MCF-7 cells (**Figure 3**). Only TNF was able to activated endothelial cells when added alone, in combination with the others cytokines or in combinations with TSFs from MCF-7 cells. The importance of TNF for endothelial cell activation has been reported: TNF is particularly important for both local and systemic inflammation, and it is a potent and well-examined activator of NF-κB in a variety of cell types, including endothelial cells [39]. Evani et al. [40] found that TNF plays an important role in the contribution of THP-1 to the arrest of MDA-MB-231 cells. In endothelial cells, a similar effect on the expression of ICAM-1 was detected, and activation of the NF-κB pathway was also found to be critical for monocyte aggregation and endothelial adhesion. Similarly, Geng et al. [41] showed that the treatment of MDA-MB-231 cell cultures with IL-6 and TNF induced growth, promoted aggregation and induced cell adhesion on E-selectin-coated surfaces. Treating MDA-MB-231, MCF-7 (breast cancer cells), MeWo and SKMEL-30 (melanoma cell) with TNF resulted in increased adhesion to lymphatic endothelium (with transgenic hTERT) and vascular endothelial cells [42]. All this suggests that TNF can serve as an important mediator of the interactions between tumor cells and endothelium.

Unexpectedly, neutralizing antibodies against TNF did not diminish the pro-adhesive phenotype or the expression of cell adhesion molecules. This led to postulate that factors (other than IFN-γ, IL-6 or IL-8) are present in TSFs from ZR 75.30 cells that can induce endothelial cell activation (**Figure 4A**). Our group recently showed that TSFs from ZR 75.30 cells treated with a neutralizing antibody against IL-1β reduced cellular adhesion modestly, indicating that this cytokine could contribute to the endothelial activation induced by TSFs from ZR 75.30 cells (**Figure 4B**).

In conclusion, we propose that TSFs from ZR 75.30 cells induce endothelial activation via the canonical NF-κB pathway and that these effects are partially due to TNF and IL-1β, supporting the concept that metastatic cells constitutively secrete pro-inflammatory factors that contribute to the recruitment of normal endothelial cells for the process of tumor cell dissemination.

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Disclosure of conflict of interest

None.

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