Immunogenic properties of renal cell carcinoma and the pathogenesis of osteolytic bone metastases

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Abstract. The immunogenic properties of renal cell carcinoma (RCC) on bone osteolysis were investigated. mRNA expression of three proinflammatory cytokines, monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), were determined in a panel of RCC lines (CRBM 1990, ACHN and Caki-1). Moreover proinflammatory cytokine mRNA expression and protein levels of adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and E-selectin, on human umbilical vein endothelial cells (HUVEC) incubated with the conditioned media from RCC lines were evaluated. RCC express mRNA of MCP-1, IL-6 and IL-8 that may induce a proinflammatory phenotype in endothelial cells. mRNA expression of IL-6, and IL-8 was induced on HUVEC treated with the conditioned media from RCC lines and mRNA and protein levels of ICAM-1 and E-selectin were also increased. This study demonstrates the immunogenic properties of renal cell carcinoma, such as pro-inflammatory cytokine secretion and the induction of adhesion molecules (ICAM-1 and E-Sel) by endothelial cells. ICAM-1 binds lymphocyte function-associated antigen-1 (LFA-1), which is expressed by pre-osteoclasts, so that, the observed proinflammatory phenotype in HUVEC may also favour osteoclast recruitment in bone metastases microenvironment. Osteolysis in bone metastases, mediated by this pathway, may be further potentiated by the pro-angiogenic properties of RCC.

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Introduction

Renal cell carcinoma (RCC) is an immunogenic neoplasm, as shown by the presence of frequent infiltration of tumor tissue with cells of the immune system (1) and a rare spontaneous regression and clinical responses to immunotherapies (2). Angiogenesis has a relevant role in the progression of the metastatic process of RCC. RCC is characterized by rich neovascularization and expression of angiogenesis-related factors, such as fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF) and matrix metallo-proteinase types 2 and 9 (3). Bone metastases from RCC are highly vascularized and predominantly osteolytic. Histological analysis has demonstrated a significant presence and activation of osteoclasts in these lesions (4). Little is known about the relationship between the inflammation process and bone resorption in this neoplasm.

Adhesion molecules for leukocytes (CAMs) are usually expressed by endothelial cells activated by inflammatory stimuli such as interleukin-1, endotoxin and tumor necrosis factor-α (TNF-α) (5). CAMs may have a role in metastases formation (6). E-selectin, involved in the adhesion of neutrophils, monocytes, and CD4^{+} 'memory' T lymphocytes to the target tissue endothelium, has also been demonstrated to mediate the rolling of prostate tumor cells to the bone marrow endothelium (7). CAMs have a role in the metastatic dissemination of renal carcinoma (6) and promote neoangio-genesis, favouring the persistence of the neoplasm (8,9). Endothelial CAM expression could favour both the adhesion of neoplastic cells and the recruitment of circulating osteoclast precursors, contributing to the pathogenesis of osteolytic metastases. CAM expression promotes osteoclast precursor recruitment to the sites of bone resorption (10,11) and in bone metabolism (12). Pre-osteoclasts, derived from haemopoietic precursors of the bone marrow and of the peripheral blood (13), express lymphocyte function-associated antigen-1 (LFA-1) (12,14), that binds ICAM-1 expressed by inflammed endothelium. The binding of vascular cell adhesion molecule-1 (VCAM-1) and α4ß1-integrin is involved in the interaction between stromal cells and osteoclast precursors (15).

Tumor cells may also induce the endothelial expression of pro-inflammatory cytokines, such as MCP-1, IL-6, and IL-8 by endothelial cells. These cytokines may also be released by the neoplastic cells in the tumor microenvironment, contributing to the recruitment of lymphocytes and monocytes, to the induction of CAMs, to the promotion of angiogenesis (16) and, in bone metastases, to the recruitment and differentiation of osteoclast precursors.

The goal of this study was to analyze the effect of a panel of RCC lines on the expression of endothelial CAMs and proinflammatory cytokines, that may be important in the pathogenesis of osteolytic bone metastases from RCC.

Materials and methods

Four separate experiments were performed for adhesion molecule on HUVEC cultures, each isolated from a different donor. In each experiment, HUVEC from a single donor were used and each sample was tested in triplicate. The cell number, evaluated by crystal violet staining after CAM-EIA, was comparable under the different conditions (data not shown).

Cell cultures. HUVEC were harvested according to the method of Jaffe *et al* (17) and grown to confluence in medium M199 (Sigma, Steinbach, Germany) supplemented with 20% fetal calf serum (FCS) (Life Technologies, Karlsruhe, Germany), 2 mM L-glutamine (Glutamax I, Life Technologies, Karlsruhe, Germany), 100 UI/ml of penicillin (Invitrogen, Carlsbad, CA, USA), 100 μg/ml of streptomycin (Invitrogen), 25 μg/ml of sodium heparin (Sigma) and 25 μg/ml of endothelial growth factor supplement (ECGS) (Becton-Dickinson, Bedford, MA) (HUVEC medium) in tissue culture flasks precoated with 0.2% gelatine in water (Sigma). Renal carcinoma bone metastasis cells (CRBM 1990), previously isolated and characterized from a bone metastasis of RCC (18), were used after 10 to 25 passages. Caki-1 and ACHN were purchased from ATCC (Manassas, VA, USA). The three human RCC lines were maintained in Ham's F12 medium modified by Coon (Sigma, St. Louis, MO, USA), supplemented with 10% FCS, 2 mM L-glutamine, 100 UI/ml of penicillin, 100 μg/ml of streptomycin (Coon's medium).

Preparation of supernatants. CRBM 1990, ACHN and Caki-1 cells were cultured for 72 h until confluency, then the conditioned media were collected, centrifuged for 5 min at 1,500 rpm to remove cell debris, aliquoted and stored at -20˚C.

Quantification of expression of cell adhesion molecules by enzyme immunoassay (CAM-EIA). HUVEC were seeded on fibronectin-coated (5 μg/ml) (Roche, Mannheim, Germany) 96-well microtiter plates (1.6x10⁴ cells/well) and after 24 h were stimulated with 50% HUVEC medium + 50% CRBM 1990, ACHN or Caki-1-conditioned media. The negative controls were both HUVEC incubated with 50% HUVEC medium + 50% Ham's F12 medium and HUVEC incubated with 100% HUVEC medium. The positive control was lipopolysaccharide (endotoxin) LPS (1 μg/ml in HUVEC medium) (lipopolysaccharide from *E. coli* 0111:B4, Sigma). The incubation time was 4 h for E-selectin, 24 h for ICAM-1, and 4 h and 24 h for VCAM-1. After incubation, HUVEC were washed twice with Dulbecco phosphate-buffered saline (DPBS) (BioWhittaker, Verviers, Belgium), fixed in methanol: ethanol (2:1) for 15 min, washed three times with DPBS and incubated with Blocking Buffer (Roche) containing 1% H_2O_2 at 37˚C for 30 min. Then the cells were incubated with the primary antibody for 45 min at 37˚C. Primary antibodies were monoclonal anti-E-selectin antibody (0.5 μg/ml, BMS 110, Bender, Vienna, Austria), monoclonal anti-ICAM-1 antibody (0.125 μg/ml, BMS 108, Bender), monoclonal anti-VCAM antibody (0.5 μg/ml, MON 6017, Monosan, Uden, The Netherlands). To check the specificity of the immunoreaction the isotype mouse IgG2a-ve was used as a control (0.5 μg/ml, CBL601, Cymbus Biotechnology, Hamburg, Germany) for E-selectin and mouse IgG1-ve (1 μg/ml, CBL600, Cymbus Biotechnology) was used as a control for VCAM-1 and ICAM-1. After three washes with DPBS the cells were exposed to the secondary antibody, antimouse IgG biotinylated 1:800 (RPN 1177, Amersham, Freiburg, Germany). Signals were enhanced by the biotin-streptavidin system (Amersham) and color reaction was performed using peroxidase-catalyzed o-phenylenediamine (Sigma). The reaction was stopped with HCl 3M. The product of the reaction was transferred in a new clean plate and the adsorbance was determined at 492 nm, while the cells were stained with crystal violet (5).

Crystal violet staining. After the CAM-EIA the cells were washed twice with DPBS and incubated with 0.1% crystal violet in water for 20 min at room temperature. Then the cells were washed with distilled water and treated with acetic acid 33% in water for 15 min. The adsorbance was determined at 600 nm.

RT-PCR analysis. HUVEC were seeded into a 6-well plate at a concentration of 4x10⁵ cells/well and suspended in serum-added medium. After 24 h at 37˚C, the medium was replaced respectively with non-conditioned medium, LPS-added medium (1 μg/ml), 50% CRBM 1990-conditioned medium, 50% ACHN-conditioned medium, 50% Caki-conditioned medium. After 6 h, total cellular RNA was isolated using Qia-shredders and RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Total cellular RNA was isolated also from ACHN, Caki-1 and CRBM 1990 at confluence using the same method. To avoid genomic DNA contamination, RNA samples were treated with Dnase I (RNase Free DNase, Qiagen GmbH, Hilden, Germany). Reverse transcription was performed using Omniscript RT kit (Qiagen GmbH). On the cDNA, PCR reactions were performed using Taq PCR Core kit (Qiagen GmbH). GenBank™ accession number of the cDNA sequences and the sizes of amplified fragments analized are: ß-actin (NM_001101; 574 bp), ICAM-1 (J03132; 395 bp), E-selectin (AF540378; 304 bp), VCAM (NM_001078; 282 bp), MCP-1 (S71513; 217 bp), granulocyte-macrophage colony stimulating factor (GM-CSF) (M13207; 196 bp), IL-8 (M28130;172 bp) and IL-6 (M14584; 173 bp). PCR primer sequences are: ß-actin (5'-AGCATTTGCGGTGGACG ATGGAG-3' and 5'-GACCTGACTGACTACCTCATGA-3'), ICAM-1 (5'-TCATCACTGTGGTAGCAGCC-3' and 5'-GTCTTGCTCCTTCCTCTTGG-3'), E-selectin (5'-TAC ACTTGCAAGTGTGACCC-3' and 5'-TGTCACAGCATCA

Table I. Expression of adhesion molecules by HUVEC incubated with the conditioned media from renal carcinomas cell cultures (arithmetic mean, standard error and significance; [a]p<0.05 vs. negative control).

|	ICAM-1 (adsorbance) 24 h	E-selectin (adsorbance) 4 h	VCAM (adsorbance)	
			4 h	24 h
Medium 199 (basal expression)	0.097±0.021	0.057±0.020	0.028±0.016	0.066±0.032
Coon's (negative control)	0.079±0.011	0.066±0.029	0.024±0.012	0.032±0.015
LPS (positive control)	1.955±0.220[a]	2.289±0.049[a]	0.689±0.183[a]	0.366±0.105
ACHN conditioned medium	0.110±0.016	0.211±0.059	0.044±0.026	0.027±0.007
Caki-1 conditioned medium	0.485±0.110[a]	0.400±0.136	0.042±0.009	0.036±0.017
CRBM 1990 conditioned medium	0.113±0.011[a]	0.259±0.097	0.062±0.035	0.034±0.005
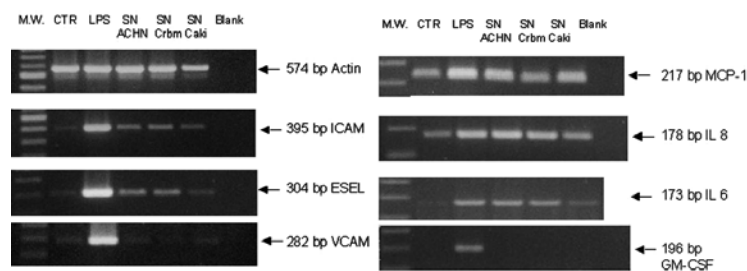
Figure 1. ICAM-1, E-selectin, VCAM-1, MCP-1, IL-8, IL-6 and GM-CSF mRNA expression by HUVEC stimulated with LPS or renal cancer cell conditioned media (representative pictures). The treatment with LPS or RCC conditioned media induced evident up-regulation of ICAM and E-selectin in HUVEC. A cytokine-specific mRNA (namely MCP-1, IL-6 and IL-8) increase was also evident in HUVEC treated with RCC conditioned media. VCAM and GM-CSF mRNA did not show any consistent variation in RCC conditioned media treated HUVEC.

CACTCAACC-3'), VCAM (5'-TCTCATTGACTTGCAGC ACC-3' and 5'-ACTTGACTGTGATCGGCTTCC-3'), MCP-1 (5'-CAGTCACCTGCTGTTATAACTTC-3' and 5'-GTGA GTGTTCAAGTCTTCGGAG-3'), granulocyte-macrophage colony stimulating factor (GM-CSF) (5'-CTGCTGCTGAGA TGAATGAAACAG-3' and 5'-GCACAGGAAGTTTCCG GGGT-3'), IL-8 (5'-CATGACTTCCAAGCTGGC CGTG-3' and 5'-CCACTCTCAATCACTCTCAGTTC-3'), and IL-6 (5'-GATGCAATAACCACCCCTGACCC-3' and 5'-CAATC TGAGGTGCCCATGCTA-3'). The PCR consisted of one cycle at 94˚C for 2 min and then 35 cycles (only for ß-actin 30 cycles) at 94˚C for 60 sec, annealing (65˚C for ß-actin and IL-8; 57˚C for ICAM-1; 52˚C for IL-6; 55˚C for MCP-1 and GM-CSF; 62˚C for E-selectin and VCAM) for 30 sec and extension at 72˚C for 30 sec, with a final extension at 72˚C for 7 min. Products were separated by electrophoresis using 1% agarose gel stained with ethidium bromide (0.5 µg/ml). DNA Ladder 100 bp was run in parallel as a molecular weight marker (Bio-Rad). The cDNA signals were normalized to ß-actin signals determined in parallel for each sample.

Immunostaining for E-selectin and ICAM-1. HUVEC were seeded on fibronectin-coated (10 µg/ml) (Roche) 8-well chamber slides (4x10⁴ cells/well) and grown for 24 h. Then the monolayers were stimulated with LPS (1 µg/ml) or 50% HUVEC medium + 50% CRBM 1990, ACHN or Caki-1-conditioned media. The negative control were HUVEC treated with 50% HUVEC medium + 50% Ham's F12 medium. The incubation times were 6 h for E-selectin or 24 h for ICAM-1.

The cells were fixed with p-formaldehyde 3.7% in PBS, then permeabilized with Hepes Triton-X 0.2% for 10 min (E-Sel) or 5 min (ICAM-1) and washed with PBS. Then the cells were incubated for 1 h with the primary monoclonal antibody anti-ELAM-1 (0.5 µg/ml, MON 6010, Monosan) 1:50 or anti-CD54 (anti-ICAM-1) 1:100 (MEM 112 Monosan). The primary antibodies were diluted in PBS + 3% bovine serum albumin (BSA) (Sigma). After three washes with PBS, the cells were exposed to the secondary antibody, goat fluorescent antimouse (A-11029, AlexaFluor, Molecular Probes, Leiden, The Netherlands) 1:1000 in PBS + 3% BSA (Sigma) for 1 h. Actin cytoskeleton was stained using rhodamine-phalloidin fluorescent dye 0.06 mM (Molecular Probes, Eugene, USA) in the dark for 30 min. The nuclei were counterstained with propidium iodide (0.5 µg/ml) (Sigma) or Hoechst Dye 33342 (1 µg/ml) (Molecular Probes).

Statistical analysis. Statistical analysis was performed with the StatView™ 5.0.1 software for Windows (SAS Institute Inc., Cary, NC). The results were reported as the arithmetic mean and standard error (mean ± SEM). The statistical analysis of the effects of conditioned media on HUVEC was made by the Student's t-test for paired samples. The level of statistical significance was set at p<0.05.

Results

ICAM-1 expression induced by RCC supernatants. Since under basal conditions there were no significant differences in ICAM-1 expression in HUVEC depending on the culture
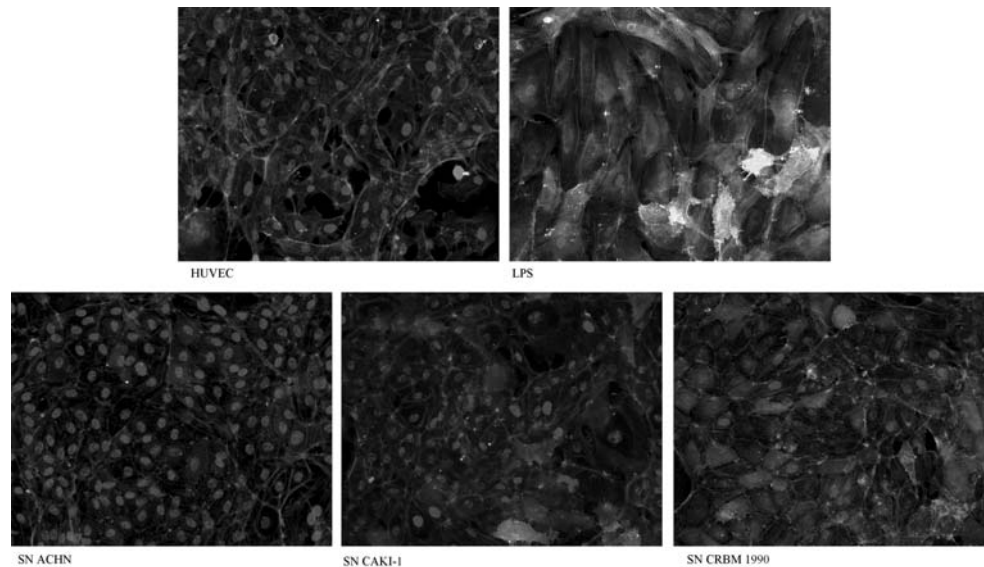
Figure 2. Effect of conditioned media from CRBM 1990, ACHN and Caki-1 on ICAM-1 expression by HUVEC (representative pictures). HUVEC were stained with endothelial cell-specific anti-CD54 and nuclei were stained with Hoechst dye. Actin cytoskeleton was stained using rhodamine-phalloidin fluorescent dye. RCC conditioned media and LPS induced a higher expression of ICAM-1 on HUVEC.
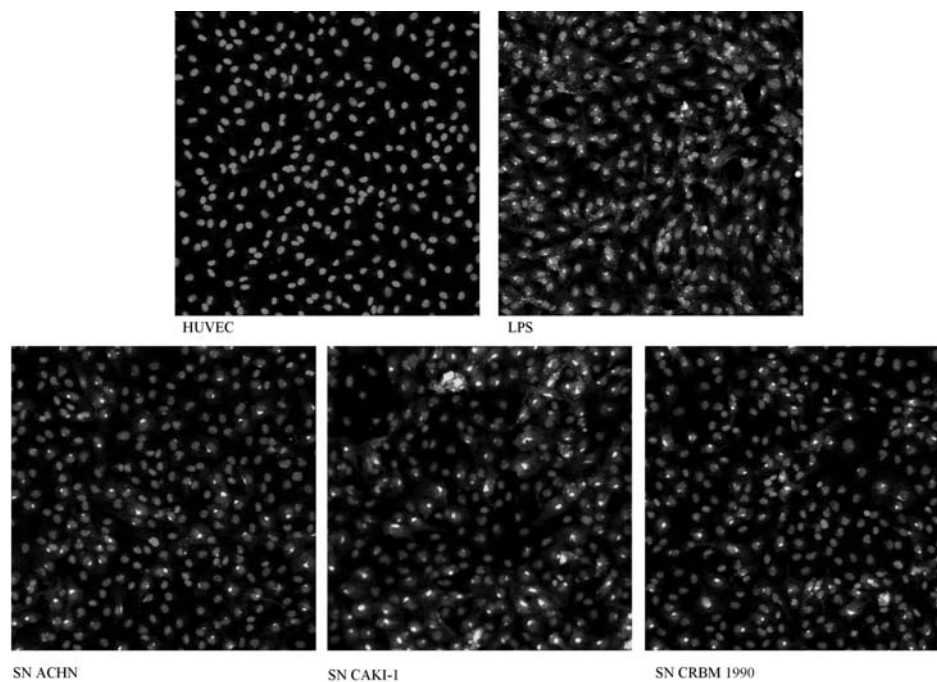

Figure 3. Effect of conditioned media from CRBM 1990, ACHN and Caki-1 on E-selectin expression by HUVEC (representative pictures). HUVEC were stained with endothelial cell-specific anti-ELAM-1 and nuclei were counterstained with propidium iodide. An evident expression of E-selectin was induced by LPS and RCC conditioned media on HUVEC.

medium (medium M199 or Coon's medium), subsequent experiments were done using Coon's medium. The stimulation with LPS induced a significantly higher ICAM-1 expression in HUVEC, in agreement to previous studies (5). After 24-h continuous exposure of HUVEC with RCC conditioned media, ICAM-1 expression was always increased compared to unstimulated culture conditions (Table I).

These findings were confirmed by RT-PCR. In fact, ICAM-1-specific mRNA was not expressed by HUVEC under basal conditions but was increased by LPS treatment. The conditioned media from ACHN, CRBM 1990 and Caki-1 induced an increase in ICAM-1 mRNA expression by HUVEC (Fig. 1). Similar results were obtained by immunostaining with ICAM-1 antibody (Fig. 2).

E-selectin expression induced by RCC supernatants. There were no significant differences in E-selectin expression in HUVEC cultured in medium 199 or Coon's medium. The stimulation with LPS induced a significantly higher E-selectin expression, in agreement to previous studies (5). Incubation
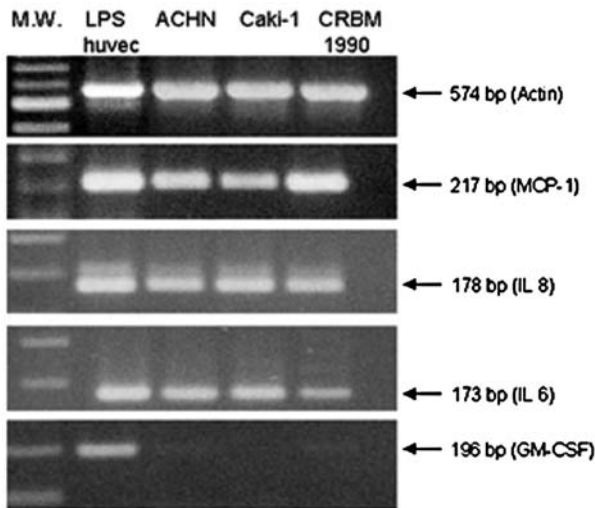
Figure 4. MCP-1, IL-8, IL-6 and GM-CSF mRNA expression by HUVEC stimulated with LPS, CRBM 1990, ACHN and Caki-1 (representative pictures). RCC cells and HUVEC stimulated by LPS showed an evident mRNA expression of MCP-1, IL-6 and IL-8, while only HUVEC stimulated by LPS produced GM-CSF.

of HUVEC with the conditioned medium from the three RCC lines always exhibited a higher level of E-selectin expression (Table I). E-selectin mRNA was not expressed by HUVEC under basal conditions but was increased after LPS stimulation. E-selectin mRNA expression was induced by the conditioned media from ACHN and CRBM 1990, but not by Caki-1 (Fig. 1). Immunostaining confirmed an increased expression of E-selectin in HUVEC treated with the supernatants of all of the three RCC cell lines (Fig. 3).

V-CAM-1 expression induced by RCC supernatants. There were no significant differences in VCAM-1 induction between

medium M199 and Coon's medium. LPS induced a significant VCAM-1 expression after 4 h, according to literature. The conditioned media from carcinomas did not induce significant variations in VCAM expression either after 4 or 24 h (Table I). As in the literature (5), VCAM-1-specific mRNA was not expressed by HUVEC under basal conditions, but was significantly induced by LPS. The conditioned media from carcinomas did not affect VCAM mRNA expression (Fig. 1).

Cytokine-specific mRNAs. By RT-PCR analysis, we observed that RCC cells produce MCP-1, IL-6, and IL-8, but not GM-CSF (Fig. 4).

As previously shown, HUVEC do not express GM-CSF or IL-6 mRNAs under basal conditions, but their expression is induced by LPS stimulation (5). The conditioned media from carcinomas did not affect GM-CSF expression. On the contrary, RCC conditioned media induced IL-6 expression in HUVEC and increased the expression of both IL-8 and MCP-1 (Fig. 1).

Discussion

The cellular mechanisms responsible for local destruction of bone by tumor cells are largely dependent on an increase in osteoclast activity, although tumor cells may directly destroy bone by proteolytic activity (19). In particular, a characteristic of bone metastases from RCC is the presence of a purely lytic process that is mediated by osteoclast recruitment and activation and accompanied by a striking neo-vascularization. These phenomena may be so evident that entire bone segments are substituted by the tumor tissue and the hypervascularity at the site of metastasis is so evident that a pulsatile mass may be seen on inspection. Little is known on the mechanisms underlying these phenomena, moreover, the exact nature of the mediators of bone destruction and increased vascularity at the metastatic site are not fully elucidated.
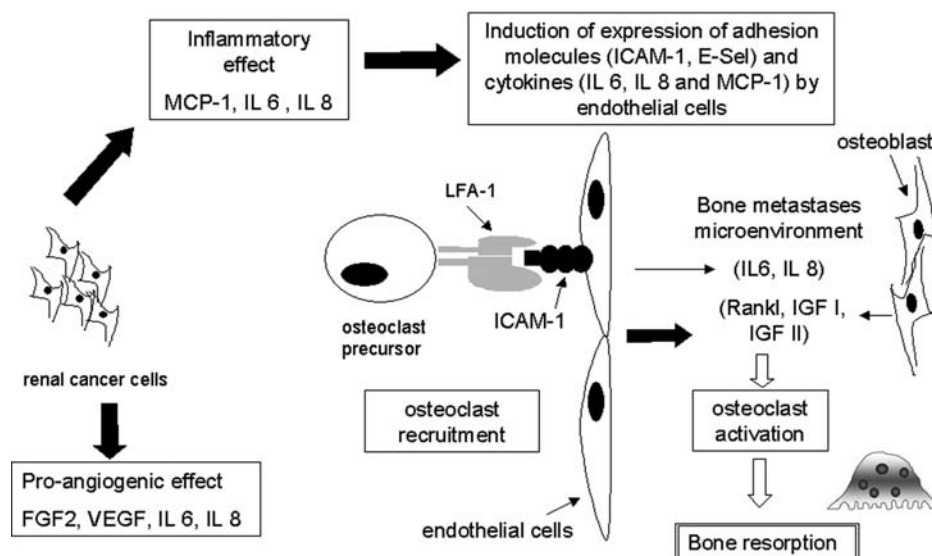

Figure 5. Schematic model of the hyphothesis of pro-inflammatory and osteolytic events in bone metastases from RCC. RCC secrete pro-inflammatory cytokines and induce the expression of CAMs, particularly ICAM-1 and E-Sel, by endothelial cells. The binding of LFA-1, expressed by osteoclast precursors, with ICAM-1, expressed by inflammed endothelium, might favour osteoclast recruitment. In bone metastases microenvironment, osteoclast activation is induced by local factors, such as IGF I, IGF II and Rankl and by IL-6 and IL-8, which are secreted by inflammed endothelium. These phenomena are potentiated by the pro-angiogenic activity of RCC.

We investigated whether RCC cells secrete soluble factors that may enhance osteoclast recruitment and neo-angiogenesis. We also studied this tumor's ability to indirectly influence osteoclasts by acting on endothelial cells. By using a panel of RCC cell lines and HUVEC as a model, we were able to demonstrate that RCC not only produce a number of pro-inflammatory mediators, but also induce the expression of adhesion molecules, namely ICAM-1 and E-selectin, in HUVEC, suggesting the existence of a multiplex paracrine influence that may be relevant for the pathogenesis of bone metastasis of this type of tumor.

Frequent infiltration of RCC tissue with cells of the immune system, as well as rare spontaneous regressions and clinical responses to cytokine therapy, suggest that since RCC is immunogenic, it may benefit from the establishment of immunotherapy (20). The expression of IL-8, a cytokine that is implicated in angiogenesis and osteoclast recruitment, is significantly higher in lymph node metastases of RCC compared to primary tumors (3). Elevated levels of IL-6, an autocrine tumor growth factor produced by RCC cells, correlated with a poor outcome in patients with metastatic RCC (21). Serum levels of IL-6 were detectable in the majority of patients with metastatic renal cell carcinoma and showed a significant correlation to progression-free survival and overall survival (22). By RT-PCR we confirmed that RCC cells produce MCP-1, IL-6, and IL-8, three cytokines that may act as mediators of the inflammatory reaction elicited by RCC in a clinical setting. The recruitment of immune cells by cytokines produced by RCC cells is mediated by the induction of adhesion molecule expression by endothelial cells (23,24). ICAM-1 mediates two important functional aspects of tumor biology, namely enhancement of tumor metastasis and mediation of host defense mechanisms such as lymphocyte-mediated tumor cytotoxicity (25). E-selectin is expressed by endothelial cells during inflammation and interact with leukocytes through sialyl-Lewis(x) and sialyl-Lewis(a) antigens. E-selectin may also be involved in tumor metastasis process, through the binding of tumor-associated glycoproteins and glycolipids, that have been reported as potential ligands for E-sel (26). High level of sialyl-Lewis(x) expression in renal cell carcinoma are crucial for tumor cell adhesion to the endothelium and metastasis (27). VCAM-1 favours monocyte and lymphocyte adhesion to endothelial cells and appears to be involved in the interaction between stromal cells and osteoclastic precursors, through the binding of α4ß1 integrin (15).

In our study, RCC cells induced the expression of two adhesion molecules, namely ICAM-1 and E-selectin, by HUVEC endothelial cells both at the molecular and protein level. The increase of ICAM-1 and E-selectin expression, induced by RCC cells, is further potentiated by its ability to induce a pro-angiogenic effect through FGF-2 and VEGF (18).

The expansion of blood vessels through angiogenesis physically enables greater delivery of circulating pre-osteoclasts into the metastases site. CAMs expressed by endothelium stimulated by tumor cells can bind osteoclast precursors and favour transendothelial migration (11). ICAM-1, expressed by the endothelium, binds lymphocyte function-associated antigen-1 (LFA-1), which is expressed by the pre-osteoclast cell membrane. ICAM-1 was shown to be an important component of the mechanism by which cytokine-pretreated HMVEC stimulated pre-osteoclasts transendothelial migration (11).

Osteoclasts activation and differentiation may be favoured by insulin growth factors I and II (IGF-I and IGF-II), two growth factors produced and stored in bone matrix (28) and receptor activator of NF-κB ligand (RANKL), secreted by osteoblasts (29,30) in bone microenvironment.

Moreover, in our study, the expression of IL-6 and IL-8, inducing osteoclast activation (31), is increased in HUVEC stimulated with conditioned media from the carcinoma lines. The induction of the expression of ICAM-1, IL-6 and IL-8 in endothelial cells may favour pre-osteoclasts recruitment then osteoclast activation, contributing to osteolysis in bone metastases from renal carcinoma (Fig. 5).

These findings support the hypothesis that the peculiar immunogenic properties of RCC activate a cascade of pro-inflammatory and osteolytic events that, in addition to pro-angiogenic effects of this tumor, may adversely affect the tumor-host interactions and lead to a clinically aggressive phenotype. Therapies aimed at the blockage of this complex network of events my be beneficial for the treatment of this tumor.

Acknowledgements

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