

Primitive and Bone Metastatic Renal Carcinoma Cells Promote Osteoclastogenesis through Endothelial Cells

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Abstract. *Background: The contribution of angiogenesis to renal carcinoma bone metastases is virtually unknown. Materials and Methods: The effect of a cell line from a renal carcinoma bone metastasis (CRBM) was compared in vitro with the primitive renal adenocarcinoma line ACHN, by evaluating the influence on the ability of bone endothelial cells to activate osteoclasts. Results: The ACHN-conditioned medium produced a significant expression of macrophage-colony-stimulating factor mRNA. The conditioned medium from ACHN, CRBM, or from endothelial cells previously stimulated with the neoplastic cell-conditioned media, had no direct effect on osteoclast differentiation from blood precursors (PBMC), such as CRBM and ACHN co-cultured with PBMC. However, PBMC co-cultured with endothelial cells previously stimulated with the CRBM-conditioned medium showed significantly higher levels of tartrate-resistant acid phosphatase. Conclusion: It is possible that the bone metastatic line CRBM releases factors that induce endothelial cells to favor osteoclast differentiation.*

Renal cell carcinoma (RCC) bone metastases are osteolytic and rich in vessels. The process of new capillary formation from pre-existing vessels is essential for tumor growth and metastasis. Endothelial cells, after stimulation with interleukin-1 or tumor necrosis factor α (TNF α), produce bone-resorbing cytokines that may also play a role in bone metastasis. The expression of osteoprotegerin and the receptor activator of nuclear factor κ B ligand (RANK-L) mRNAs was demonstrated in human microvascular

endothelial cells (1). If the tumor cells, in addition to angiogenesis, also induced the expression of an osteolysis-favoring phenotype, bone metastases could be favored. Only a few studies have investigated the relationship between RCC, endothelial cells and osteoclasts.

Our aim was to study the osteoclast-activating effect of a cell line from an RCC bone metastasis and to compare it with a line of a primitive renal adenocarcinoma.

Materials and Methods

Cell cultures. Renal carcinoma bone metastasis (CRBM) cells were isolated from an RCC bone metastasis (2), and were used after 10 to 20 passages. The human renal adenocarcinoma cell line ACHN was purchased from ATCC (Manassas, VA, USA). The bovine bone endothelial cell line (BBE) was originally cloned from fetal bovine sternum (3). The lines were maintained in Ham's F12 medium modified by Coon (Sigma, St. Louis, Missouri, USA).

Endothelial cell gene expression. The endothelial cells were seeded in a 12-well plate at a density of 5×10^4 cells/well, suspended in complete medium. After 24 h at 37°C, the medium was replaced respectively with non-conditioned medium, 50% CRBM-conditioned medium, 50% ACHN-conditioned medium or TNF α (4 ng/ml) in non-conditioned medium. All samples were assayed in triplicate. After 24 h at 37°C, the RNA was isolated using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Total RNA was reverse transcribed into cDNA using an Advantage RT-for-PCR Kit (Clontech Laboratories Inc., Palo Alto, CA, USA). Semi-quantitative PCR for bovine interleukin-6 (IL-6), macrophage-colony-stimulating factor (M-CSF) and β -actin were performed using forward and reverse primers from the GenBank™ (Table I). The images of the gel were quantified by a software designed for the densitometric evaluation of the bands (Quantity One, BioRad, Hercules, CA, USA). The IL-6 and M-CSF signals were normalized to β -actin signals determined in parallel for each sample and the data were expressed as an IL-6/ β -actin or M-CSF/ β -actin ratio. Each experiment was repeated 3 times.

Osteoclast development from human peripheral blood mononuclear cells (PBMC). CRBM, ACHN or endothelial cells were seeded in

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Table I. Oligonucleotides of 5' and 3' primers and PCR conditions.

GeneBank sequence	5' primer	3' primer	Position	PCR conditions					
				Pre-denaturation	Cycle n°	Denaturation	Annealing	Extension	Final extension
IL-6 (X_57317)	5'GAACCTCCGC TTCACAA3'	5'CAAGCAAAT CGCCTGAT3'	45-362	-	30	94°C 45 s	49°C 30 s	72°C 25 s	-
M-CSF (D_87918)	5'AGAGGCAGC ACAAGGAA3'	5'GTCCTCTTCC TGGGTCA3'	551-734	-	30	94°C 45 s	49°C 30 s	72°C 60 s	-
β-actin (NM_173979)	5'CCAACCGTGA GAAGATGA 3'	5'GAGGTAGTC CGTCAGGT 3'	341-568	94°C 5 min	30	94°C 30 s	54°C 30 s	72°C 30 s	72°C 7 min

the upper compartment of polycarbonate filters (Transwells, Costar) with a pore size of 0.4 µm, at a density of 3x10³ cells/well. After 5 h, the media of endothelial cells on the polycarbonate filters were replaced respectively by non-conditioned medium, 50% CRBM-conditioned medium, 50% ACHN-conditioned medium or TNFα (4 ng/ml) in non-conditioned medium. The cultures were incubated at 37°C for 48 h. Human PBMC from buffy coats of blood anticoagulated with CPDA were isolated on a Ficoll-Hystopaque gradient (Hystopaque, Sigma), resuspended in D-MEM high glucose medium (Euroclone, Wetherby, West York, UK), with 10% FBS N.A. (Pierce, Rockford, IL, USA) (non-differentiating medium) and seeded in the lower compartment of the polycarbonate filters (co-cultures with CRBM, ACHN and BBE) at a density of 8.4x10⁵ PBMC/well. Other PBMC were seeded in the wells of a 96-well microplate, at the same cell density, and treated after 24 h (day 1) according to the following protocol:

- differentiating medium (D-MEM high glucose supplemented with 10% FBS N.A., 10⁻⁷ M PTH (Sigma), 30 ng/ml RANK-L (Peprotech, Rocky Hill, NJ, USA), and 25 ng/ml M-CSF (Peprotech) (positive control);
- non-differentiating medium + CRBM-conditioned medium (1:1 ratio);
- non-differentiating medium + ACHN-conditioned medium (1:1 ratio);
- non-differentiating medium + endothelial cell-conditioned medium (1:1 ratio);
- non-differentiating medium + conditioned medium (1:1 ratio) from endothelial cells previously stimulated with CRBM medium;
- non-differentiating medium + conditioned medium (1:1 ratio) from endothelial cells previously stimulated with ACHN medium;
- non-differentiating medium + conditioned medium (1:1 ratio) from endothelial cells previously stimulated with TNFα.

CRBM, ACHN and BBE were also seeded in the wells of a 96-well microplate, at a density of 3x10³ cells/well. On day 8, the filters were removed and the PBMC in the lower compartment, as well as the cells on the microplate, were lysed with Triton-X, acidified with 0.8 M acetic acid and assayed for tartrate-resistant acid phosphatase (TRAP) activity (ACP, Roche, Mannheim, Germany). Reagent mixture (0.25 ml) was added to 0.02 ml of each lysate, the samples were incubated at 37°C for exactly 30 min and the absorbance was read at 405 nm. The sample concentration was extrapolated from a standard curve by using scalar dilutions in

Table II. Mean±SEM of IL-6/β-actin and M-CSF/β-actin ratios expressed by bovine bone endothelial (BBE) cell cultures.

BBE cell treatment	IL-6/β-actin ratio	M-CSF/β-actin ratio
Non-conditioned medium	0.25±0.063	0.16±0.021
50% conditioned medium from CRBM	0.17±0.090	0.19±0.065
50% conditioned medium from ACHN	0.17±0.042	0.24±0.031*
TNFα (ng/ml)	0.43±0.154	0.39±0.046*

*p<0.05 vs. non-conditioned medium.

Triton-X of a sample at a known TRAP concentration (C.f.a.s., Roche). The quantification of total proteins in the lysates was performed by the BCA Protein Assay Reagent Kit (Pierce). The TRAP activity of each sample was expressed as the ratio IU TRAP/µg proteins. Each experiment was repeated 5 times.

Statistical analysis. The statistical analysis was performed with the StatViewTM 5.0.1 software for Windows (SAS Institute Inc., Cary, NC, USA). Data were presented as the mean ± standard error. For the evaluation of mRNA expression, the comparison among the single treatments of cells and the negative control (*i.e.*, cells cultured in non-conditioned medium) was made by the Student's *t*-test. With regards to the TRAP assay, the differences between treatments were analyzed using the Wilcoxon test. The level for significance was set at p<0.05.

Results

Endothelial cell gene expression. The endothelial cells expressed IL-6-specific mRNA at the basal level; an increase of 1.7 times the basal level was induced by

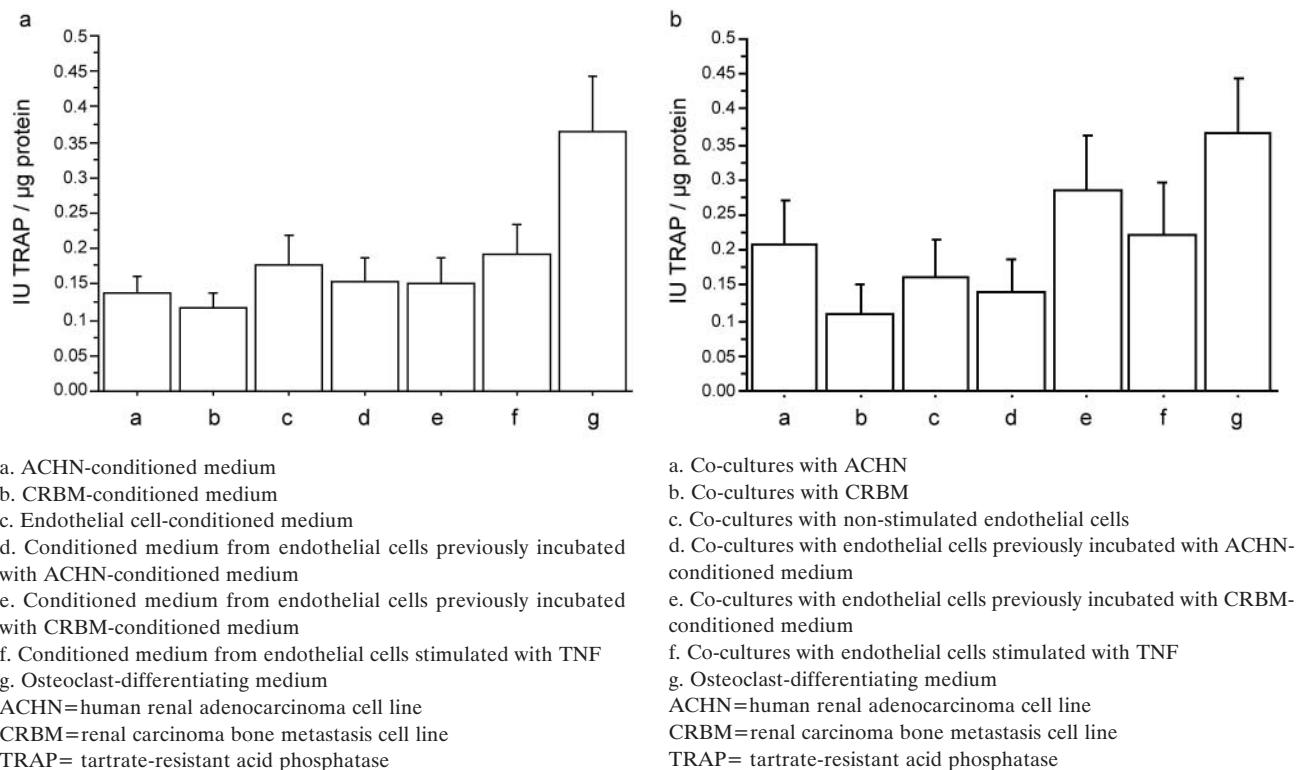


Figure 1. a. Mean \pm standard error of TRAP, determined as IU TRAP / μ g proteins, in the lysates of PBMC after 8-day incubation with the conditioned media of ACHN, CRBM or endothelial cells. b. Mean \pm standard error of TRAP, determined as IU TRAP / μ g proteins, in the lysates of PBMC co-cultured for 8 days with ACHN, CRBM or endothelial cells.

stimulation with TNF α (positive control). Incubation with CRBM- and ACHN-conditioned media did not produce significant variations in IL-6 mRNA expression. M-CSF mRNA was expressed by the endothelial cells at the basal level; TNF α induced a significant increase of 3.9 times the basal level. Incubation with CRBM-conditioned media did not produce significant variations in expression, whereas ACHN-conditioned medium induced a significant increase in expression ($p<0.05$) (Table II).

Osteoclast development in human peripheral blood mononuclear cells (PBMC). CRBM, ACHN and endothelial cells were negative for TRAP activity. The highest TRAP level in the PBMC cultures was induced by the differentiating medium. There were no significant differences among the PBMC incubated with the various conditioned media (Figure 1a). The co-cultures of PBMC with CRBM, with non-stimulated endothelial cells or with endothelial cells previously stimulated with the ACHN-conditioned medium had the lowest TRAP levels. Non-significantly higher TRAP levels were demonstrated in the co-cultures of PBMC with ACHN or with endothelial cells

stimulated with TNF α . In contrast, PBMC co-cultured with endothelial cells previously stimulated with the CRBM-conditioned medium had significantly higher TRAP levels than PBMC co-cultured with CRBM ($p<0.05$). The TRAP levels of the co-cultures with endothelial cells previously stimulated with the CRBM-conditioned medium were also significantly higher than those of the PBMC incubated with the conditioned media from endothelial cells non- or previously stimulated by CRBM or by ACHN ($p<0.05$) (Figure 1b).

Discussion

This study was a preliminary investigation into the relationships between renal tumor, angiogenesis and osteoclast differentiation *in vitro*. The activity of a cell line isolated from a bone metastasis of renal carcinoma was compared with that of a primitive continuous line (ACHN). An endothelial line isolated from fetal bovine sternum was also tested, since endothelium has different properties according to different parts of the body (4). The properties of this bovine cell line have been reported in several studies

(5, 6). Previously, it was demonstrated that CRBM- and ACHN-conditioned media induced a significant endothelial cell migration and proliferation, connected to angiogenic factors produced by neoplastic cells, such as VEGF, TGF- β and FGF-2 (2).

Since bone metastases of renal carcinomas are well vascularized and rich in osteoclasts, the carcinoma could stimulate the recruitment of osteoclasts, directly or indirectly through endothelial cells. Neither CRBM nor ACHN expressed RANK-L (2). Several angiogenic growth factors released from renal carcinomas promote osteoclast recruitment, commitment and differentiation. Flt-1, the receptor for VEGF, is expressed by peripheral blood monocytes (7). VEGF induces chemotaxis of osteoclast precursors (8) and increases the area of bone resorption pits *in vitro* (9). FGF-2 determines osteoclast differentiation by an indirect pattern *via* osteoblasts to induce RANKL/ODF through COX-2 induction (10). Actually, it appears that FGF-2 does not stimulate osteoclast development from bone marrow mononuclear cell precursors, but acts on mature osteoclasts by activating bone pit resorption (11). TGF- β enhances osteoclastogenesis induced by RANK-L (12), enhancing, in particular, multinucleated osteoclast-like cell formation, the expression of TRAP and c-src (13).

With regard to the effect on osteoclastogenesis mediated by the endothelium, the close physical interaction among endothelial cells, pre-osteoclasts and osteoclasts during bone resorption permits endothelial cells to influence the regulatory signals for osteoclast development. Molecules expressed on the endothelial cell membrane may activate circulating pre-osteoclasts during their transmigration across blood vessels, in response to local stimulatory signals, to reach the bone microenvironment. It has also been shown that endothelial cells of the microcirculation express RANK-L, if stimulated by pro-inflammatory cytokines (1).

We investigated whether neoplastic cells isolated from bone metastasis induce the endothelium to express pro-osteolytic functions. After stimulation with CRBM- and ACHN-conditioned media, IL-6 expression in endothelial cells was not increased. The ACHN-conditioned medium, but not the CRBM-conditioned medium, induced a significant increase in M-CSF expression in endothelial cells, albeit to a lesser extent than TNF α . M-CSF induces macrophage recruitment, which differentiate into osteoclasts.

Concerning the effect on osteoclast differentiation, the TRAP assay enabled us to assess the behavior of PBMC co-cultured with neoplastic or endothelial cells or incubated with their conditioned media. The neoplastic cells, ACHN or CRBM, had no TRAP activity by themselves. Their conditioned media also had little effect on the induction of TRAP activity in PBMC. TRAP activity was increased by the co-culture of PBMC with ACHN or with endothelial cells, but not with CRBM. TRAP activity was further

increased by the co-culture with endothelial cells previously stimulated with medium conditioned by CRBM, but not by ACHN.

The differentiation effect of bovine bone endothelial cells with regards to osteoclast progenitors had already been demonstrated in the past (4). The stimulation of BBE with CRBM-conditioned medium, however, caused the induction of this phenomenon to a significantly greater extent. Therefore, we may suppose that endothelial cells activate the differentiation of osteoclasts and that such activity is further increased by cells from bone metastases. It can be supposed that CRBM release a factor into the medium that induces endothelial cells to express factors activating osteoclasts. Such an effect is not present in ACHN. This factor might be a growth factor, such as TGF- β , that has been shown to up-regulate the expression of RANK-L in bone marrow-derived endothelial cells and in vascular endothelial cells, but not in osteoblasts (14), or pro-inflammatory cytokines, such as TNF α and IL-1 β , that induce endothelial cells to express RANK-L.

In conclusion, the cells from a bone metastasis of a renal carcinoma investigated in this study had little direct effect on osteoclast differentiation. It could be supposed that these cells act through the endothelium in different ways. ACHN induced M-CSF expression by endothelial cells; CRBM, isolated from an osteolytic metastasis, released factors into the medium that induced endothelial cells to favor the differentiation of osteoclasts.

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