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Isolation and Characterization of a New Cell Line from a Renal Carcinoma Bone Metastasis

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Abstract. *Background: The use of cell lines isolated from metastases should enable the assessment of peculiar aspects of bone resorbing cytokine expression, as well as angiogenetic activity of renal carcinoma bone metastases. Materials and Methods: A cell line (CRBM-1990) was isolated from a renal cell carcinoma bone metastasis and compared with the ACHN and Caki-1 established lines. The expression of osteolytic cytokine and angiogenetic growth factors mRNAs, as well as the effect on migration and proliferation of a bovine bone cell line (BBE) were determined. Results: There were no significant differences between the three lines in IL-6, TGF-β, VEGF-A, VEGF-B, VEGF-C and FGF-2 mRNAs expression. VEGF-D, PlGF, or RANK-L-specific mRNA were not expressed. CRBM-1990-, Caki-1- and ACHN-conditioned media significantly stimulated the migration and proliferation of BBE. Conclusion: CRBM-1990 expressed IL-6-specific mRNA, but not RANK-L, expressed angiogenetic growth factors and induced migration and proliferation of bone endothelial cells at a non-significantly different level when compared with Caki-1 and ACHN.*

The major cause of death from renal cell carcinoma is metastases that commonly develop in the body skeleton, with purely osteolytic lesions, which are osteoclast-rich and highly vascularized. They may cause pathological fractures, severe pain and a dramatic reduction in the quality of life. Metastasis to the spine may cause neurological symptoms secondary to cord compression.

Bone metastases depend on colonization and growth of the tumor cells in the bone compartment, bone destruction by activated osteoclasts and direct bone destruction by metastatic cancer cells. Local factors, such as growth factor and cytokines, may influence the metastatic dissemination of neoplastic cells.

In this research, the behavior of a cell line newly isolated from a renal cell carcinoma bone metastasis was compared with an established cell line from a primitive renal adenocarcinoma and an established cell line from a skin metastasis of renal clear cell carcinoma, in order to investigate the peculiar aspects of growth factor and bone resorbing cytokine expression, as well as angiogenetic activity.

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Key Words: Bone metastasis, renal cell carcinoma, osteolysis, angiogenesis.

Materials and Methods

Human tissue harvesting and processing. CRBM-1990 was developed from a 75-year-old male with metastatic renal cell carcinoma in the thoracic vertebral body T11. The patient signed a consent form allowing the collection of tissue specimens and clinicopathological data. The metastatic tissue was minced into fragments, which were seeded in Iscove's Modified Dulbecco's Medium (Sigma, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum (FBS) (Mascia Brunelli, Milan, Italy) and incubated at 37°C. The colonies with a typical morphology of renal carcinoma were isolated and amplified.

Established cell lines. Two established cell lines of human renal carcinoma, purchased from ATCC (Manassas, VA, USA), were tested: Caki-1, isolated from a skin metastasis of renal clear cell carcinoma, and ACHN, isolated from a primitive renal adenocarcinoma. The cell lines were maintained in Ham's F12 medium modified by Coon (Sigma), supplemented with 10% FBS.

RT-PCR analysis. CRBM-1990, Caki-1 and ACHN cells were cultured in Ham's F12 medium modified by Coon, in 75-cm^2 flasks. At confluency, RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcripted into cDNA using Advantage RT-for-PCR Kit (Clontech Laboratories, Palo Alto, CA, USA). Semi-quantitative RT-PCR amplification for vascular growth factor-A (VEGF-A), VEGF-B, VEGF-C, VEGF-D, PlGF, fibroblast growth factor-2 (FGF-2), interleukin-6 (IL-6), transforming growth factor-β1 (TGF-β1) and receptor activator of nuclear factor Î B ligand (RANK-L) were performed using primers from the GenBank™ (Table I). Parallel reactions were performed

Table I. *Oligonucleotides of 5' and 3' primers.*

	5' primer	3' primer	GenBank sequence	position	product length (bp)
β-actin	ATCTGGCACCAC ACCTTCTACAAT GAGCTGCG	CGTCATACTCC TGCTTGCTGAT CCACATCTGC	NM_001101	326-1164	838
VEGF-A	CTTCCAGGAGTA CCCTGA	GCCTCGGCTTG TCACA	NM_003376	1221-1682	461
VEGF-B	TTAGGTCTGCAT TCACACTG	CCAGAGGAAA GTGGTGTCAT	NM_003377	142-430	288
VEGF-C	TAGATGTGGGGA AGGAGTT	ATCGGCAGGA AGTGTGATT	NM_005429	843-1062	219
VEGF-D	AGAATGGCAAAG AACTCAGT	GCAAGCACTTA CAACCTGT	NM_004469	821-1087	266
FGF-2	TCAGACCTCTAC TGTCA	CTGGTGCCACG TGAGA	NM_002006	4528-5043	515
PlGF	CTGGGAACGGCT CGTC	CGCAGCAGGGA GACACA	NM_002632	447-601	154
IL-6	ATGAACTCCTTC TCCACAAGCGC	GAAGAGCCCTC AGGCTGGACTG	NM_000600	63-690	628
TGF-β1	GCCCTGGACACC AACTATTGCT	AGGCTCCAAAT GTAGGGGCAG G	M38449	1-161	161
RANK-L variant 1	CGTCGCCCTGTT CTTCTA	GAGTTGTGTCT TGAAAATCTGC	NM_003701	345-466	122

for every assay using primers designed to amplify human β-actin. Specific cDNA for VEGF-A was determined by denaturation at 94˚C for 10 minutes and then 30 cycles of amplification of denaturation at 94˚C for 60 seconds, annealing at 55˚C for 30 seconds and extension at 72˚C for 45 seconds, before final extension at 72˚C for 7 minutes. Specific cDNA for VEGF-B was determined by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 60˚C for 30 seconds and extension at 72˚C for 30 seconds, before final extension at 72˚C for 7 minutes. Specific cDNA for VEGF-C and VEGF-D was determined by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 56˚C for 30 seconds and extension at 72˚C for 30 seconds, before final extension at 72˚C for 7 minutes. Specific cDNA for FGF-2 was determined by denaturation at 94˚C for 10 minutes and then 30 cycles of denaturation at 94˚C for 60 seconds, annealing at 51˚C for 30 seconds and extension at 72˚C for 45 seconds, before final extension at 72˚C for 7 minutes. Specific cDNA for PlGF was determined by denaturation at 94˚C for 5 minutes and then 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 54˚C for 30 seconds and extension at 72˚C for 30 seconds, before final extension at 72˚C for 7 minutes. Specific cDNA for IL-6 and TGF-β1 was determined by 30 cycles of denaturation at 94˚C for 25 seconds, annealing at 60˚C for 45 seconds and extension at 72˚C for 2 minutes, before final extension at 72˚C for 7 minutes. Specific cDNA for RANK-L was determined by denaturation at 95˚C for 5 minutes and then 35 cycles of denaturation at 94˚C for 30 seconds, annealing at 54˚C for 30 seconds and extension at 72˚C for 30 seconds, before final extension at 72˚C for 10 minutes. Specific cDNA for β-actin mRNA levels was assayed by denaturation at 94˚C for 10 minutes and then 30 cycles of denaturation at 94˚C for 45 seconds, annealing at 65˚C for 90 seconds and extension at 72˚C for 120 seconds, before final extension at 72˚C for 7 minutes. Products were separated by electrophoresis using 2% agarose gel stained with ethidium bromide (0.5 μg/ml). DNA ladder 100 bp was run in parallel as a molecular weight marker (New England Biolabs, Beverly, MA, USA). The images of the gel were transferred to the computer by a videocamera and quantified by dedicated software for densitometric evaluation of the bands (Quantity One, BioRad, Hercules, CA, USA). The specific bands were normalized to β-actin signals determined in parallel for each sample.

MET antigen analysis. The cells were lysed with a boiling solution made of one part of 10% SDS, one part of 0.5M Tris-HCl pH 6.8 and two parts of water. The lysates were boiled for three minutes and sonicated three times for fifteen seconds. The absorbance of the samples was read at 562 nm. Using a standard curve, a preparation of diluted albumin standards (BSA), the protein concentration was determined in μg/ml for each unknown sample (1). Cell lysates (60 μg/lane) were subsequently electrophoresed on 8% polyacrylamide gel and transferred to nitrocellulose sheets. Blots were probed with the anti-met receptor polyclonal antibody C-12 (Santa Cruz, Santa Cruz, CA, USA) 1:1000 for 1 hour 30 minutes and then with horseradish peroxidase-conjugated anti-rabbit immunoglobulins (Amersham, Little Chalfont, Buckinghamshire, England) 1:1000 for 30 minutes. The signal was visualized using a chemiluminescence detection system after 5 minutes of exposure.

RANK-L protein analysis. The expression of cytoplasmic RANK-L and membrane was assessed by Western blot. Cell lysates (60 μg/lane) were electrophoresed on a 10% polyacrylamide gel and transferred to nitrocellulose sheets. As a standard control we used 0.02 mg of human RANK-L (PeproTech, Rocky Hill, NJ, USA). Blots were probed with the anti-RANK-L biotinylated (PeproTech) 1:250 for 1hour 30 minutes and then with anti-rabbit-HRP (Amersham) 1:1000 for 30 minutes. The signal was visualized using a chemiluminescence detection system after 15 minutes of exposure.

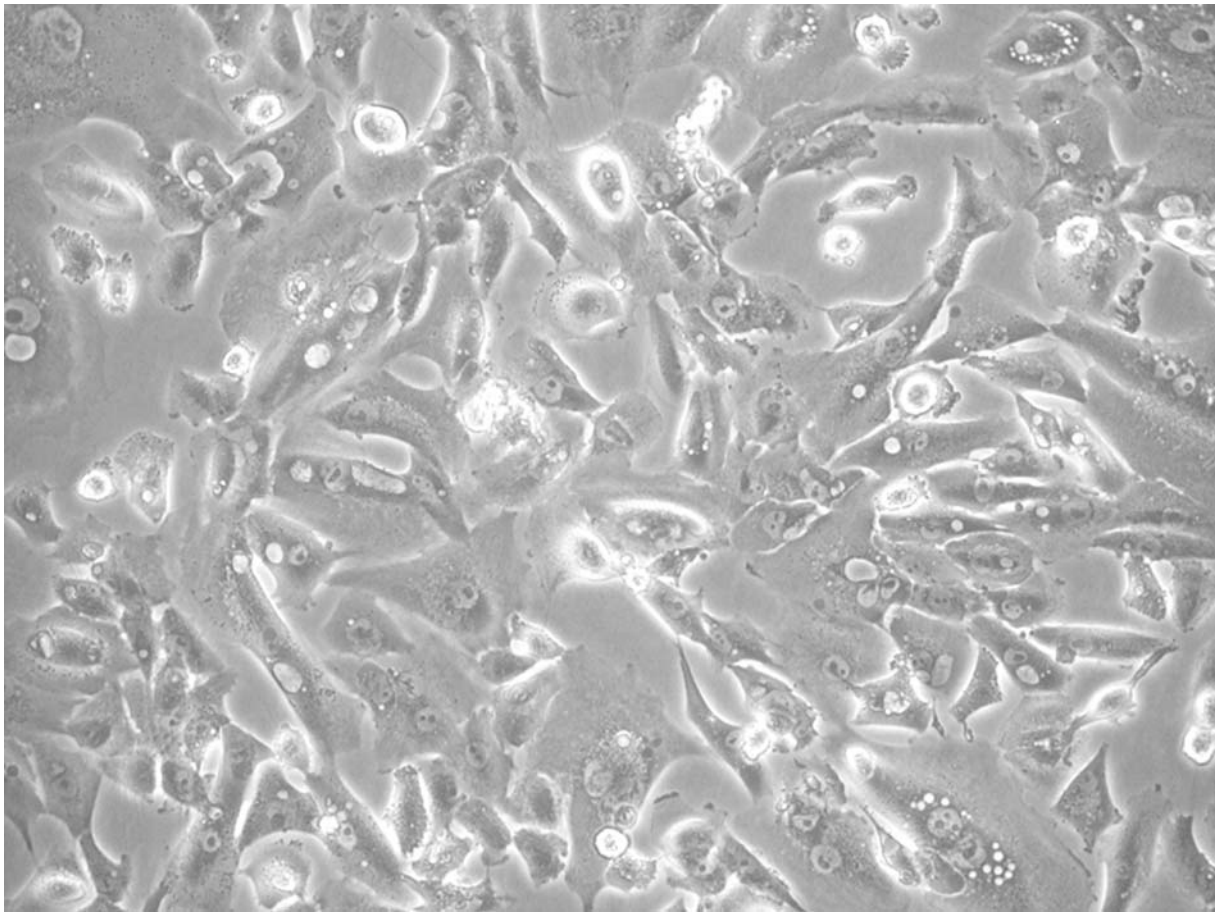

Figure 1. *Single layer of the cells isolated from the bone metastasis of the renal cell carcinoma (unfixed cell culture, 20x).*

The presence of the RANKL protein on the cell membrane was detected by flow cytometry. A cell suspension ($1x10^6$/ml) was incubated with 0.2 μg/ml of monoclonal antibody specific for the extracelluar domain of RANKL protein (Alexis Corporation, Lausen, Switzerland) and stained with 10 μl of FITC conjugated goat anti-mouse immunoglobulins (DakoCytomation, Daki, Glostrup, Denmark). A flow cytometer equipped with argon laser (EPICS XL-MCL, Beckman Coulter, Hialeah, FL, USA) was used for the analysis. The percentage of fluorescent cells was calculated out of 10,000 events.

For the RANKL detection in the conditioned medium the following antibodies were used (PeproTech): goat anti-human RANKL (2 μg/ml diluted in carbonate/bicarbonate buffer, pH 9.5) and biotinylated goat anti-human RANKL (0.2 μg/ml). The standard reference curve was set by using recombinant human sRANKL (PeproTech) with a range between 0.01 and 50 ng/ml. 96-well microplates were coated overnight with 100 μl of goat anti-human sRANKL. After washing, each well was blocked with 300 μl of PBS containing 4% bovine serum albumin (BSA) and incubated for one hour at room temperature. One hundred μl of each sample and serial dilutions of the recombinant sRANKL were incubated for two hours at room temperature. Binding between RANKL and antibody was detected by the following sequence of reactions: anti-cytokine biotinylated antibody, streptavidin-peroxidase conjugate and the substrate reacting with peroxidase, namely tetramethylbenzidine. The reaction was stopped with 0.18M H_2SO_4 and the optical density read at 450 nm wavelength The detection limit was 0.05 ng/ml.

Angiogenesis evaluation. CRBM-1990 cells were used after 10 to 20 passages. The angiogenetic effect of CRBM-1990, Caki-1 and ACHN cell lines was tested on the bovine bone endothelial cell line (BBE), originally cloned from fetal bovine sternum (2). The cell lines were maintained in Ham's F12 medium modified by Coon. CRBM-1990, Caki-1 and ACHN cells were cultured till confluency, then the supernatants were collected, centrifuged at 3000 r.p.m. for 5 minutes and stored at -20˚C (conditioned media).

In chemotaxis assays the conditioned media were assayed undiluted. The chemotaxis assay on BBE was performed with the Boyden chamber technique using a 24-well plate. In the lower compartment of the wells, serum-free medium (0.8 ml), or undiluted CRBM-1990-, Caki-1-, or ACHN-conditioned medium (0.8 ml) were placed. BBE were resuspended in serum-free medium, at a final density of 1 x 10^5 cells/ml. After placing polycarbonate filters (Transwells, Costar, Corning, Acton, MA, USA) with a pore size of 8 μm over the wells, 200 μl of the cell suspension were seeded in the upper compartment. Cells were allowed to migrate for 5 hours at 37˚C in a humidified atmosphere with 5% CO_2. Then the filter was removed and migrated cells on the lower side were fixed in 11% glutheraldheyde, stained with crystal violet solution and counted from nine random fields (magnification x20) in each well.

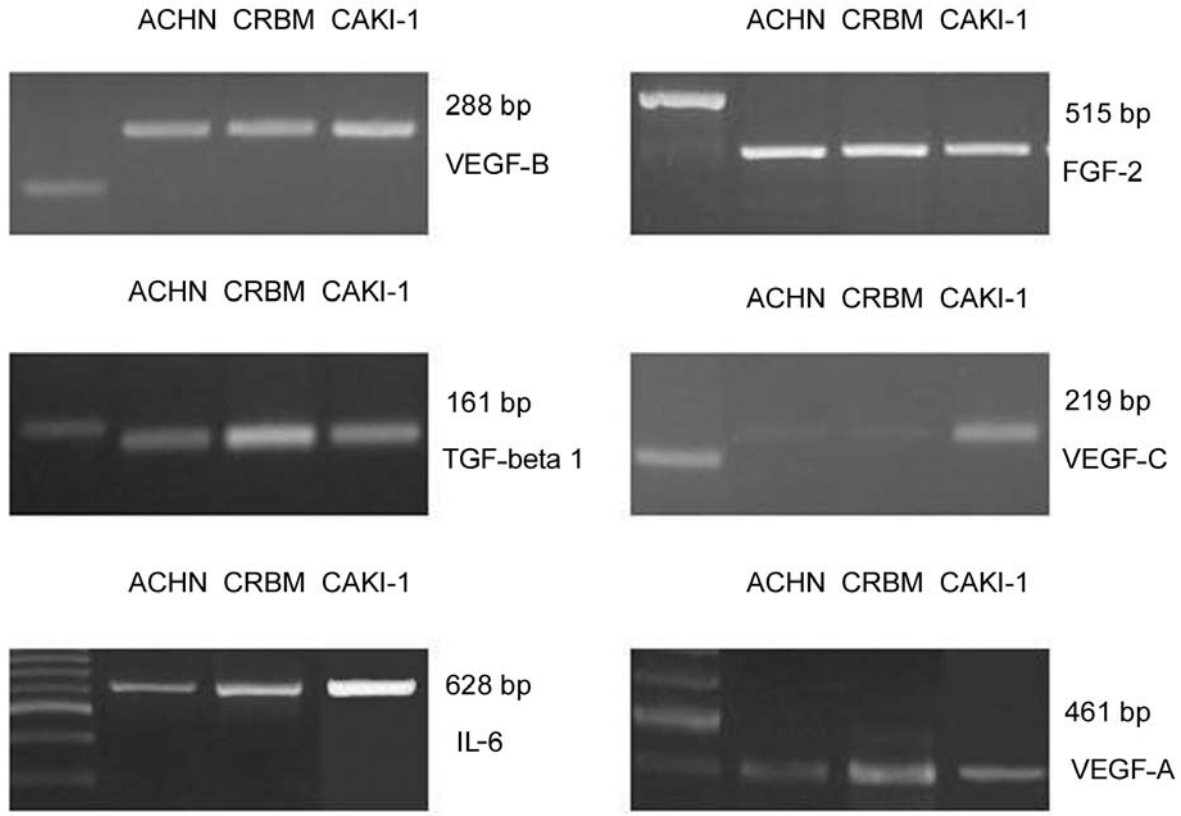

Figure 2. *mRNA expression for angiogenetic and osteolytic cytokines.*

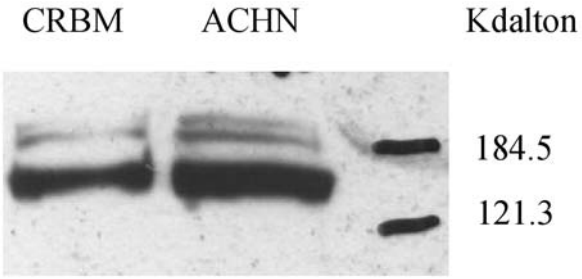

Figure 3. *Expression of MET by Western blot.*

For the growth assay, BBE (1 x 10^4) were plated in 12-well plates (Costar) in complete medium. After 24 hours the cells of two wells were detached and counted in duplicate (time 0). In the remaining wells the medium was removed and replaced respectively with non-conditioned medium, or 50% CRBM-1990-, Caki-1-, or ACHN-conditioned medium. Each sample was tested in duplicate. After 24, 48, 72 and 96 hours, the BBE cells were counted in duplicate.

Statistical analysis. Statistical analysis was performed with the StatView™ 5.0.1 software for Windows (SAS Institute Inc., Cary, NC, USA). Data were presented as the mean ± standard error. The differences between the couples of the samples were tested by the Mann-Whitney *U*-test. The level for significance was set at $p<0.05$.

Results

Morphology of the isolated cells. Histology of the bone metastasis showed poorly-differentiated carcinoma, consistent with renal cell carcinoma. The cells isolated with the culture had an epithelial morphology, with nuclei of varying sizes. Several cells in mitosis were detected, whereas very few were in apoptosis (Figure 1).

RT-PCR analysis. Three RNA separated isolates for each line were tested. There were no significant differences with regards to the expression of the house-keeping gene β-actin, mRNA specific for proangiogenetic growth factors VEGF-A, VEGF-B, VEGF-C, FGF-2 and TGF-β1, or that specific for osteolytic cytokine IL-6 (Figure 2 and Table II). None of the three cell lines expressed mRNA specific for VEGF-D, PlGF, or RANK-L. No products were obtained in controls lacking cDNA.

MET antigen analysis. The antigen MET is expressed by two bands: Pre-MET of 175 Kda and MET of 140 Kda. Both bands were expressed by CRBM-1990 cells and ACHN cells (Figure 3).

Table II. *Mean±SEM of β-actin cDNA density and growth factors or cytokines/β-actin ratios.*

mRNA	CRBM-1990	Caki-1	ACHN
β-actin density (INT/mm^2)	550.784±18.894	291.986±30.571	510.158±26.308
VEGF-A/β-actin ratio	0.472±0.032	0.976±0.781	0.133±0.042
VEGF-B/β-actin ratio	0.640±0.017	1.525±0.140	0.537±0.113
VEGF-C/β-actin ratio	0.254±0.004	1.381±0.618	0.359±0.166
FGF-2/β-actin ratio	0.711±0.095	1.344±0.141	0.904±0.147
TGF-β1/β-actin ratio	1.156±0.217	1.394±0.617	0.582±0.214
IL-6/β-actin ratio	0.946±0.311	2.143±0.207	0.810±0.236

RANK-L protein expression. On Western blot, RANK-L is normally shown by a band of 35 KDalton; the recombinant antigen also has a band of 20 KDa. It was expressed neither by CRBM-1990, nor by ACHN. Flow cytometric evaluation showed the lack of the cell membrane form of RANKL in both CRBM-1990 and ACHN and the immunoenzymatic method did not detect the presence of soluble RANKL.

Angiogenesis evaluation. Four independent experiments were performed for chemotaxis assessment; in each experiment each sample was tested in quadruplicate. CRBM-1990-conditioned medium significantly stimulated the migration of BBE compared with the non-conditioned medium (negative control) (42.8±11.6 migrated BBE cells for CRBM-1990-conditioned medium; 8.8±1.1 migrated BBE cells for non-conditioned medium; $p<0.01$), such as Caki-1- (97.2±14.8 migrated BBE cells; $p<0.01$) and ACHN-conditioned media (68.3±13.4 migrated BBE cells; $p<0.01$) (Figure 4).

Each experiment of the BBE growth assay was repeated four times. Before adding the conditioned media, the number of cells in the wells was 10.301±1.272 x10^3. BBE cells incubated with CRBM-1990-conditioned medium showed a faster growth rate compared to the cells cultured with non-conditioned medium only (CRBM-1990 24h: 25.181±3.629 x10^3 *vs*. non-conditioned medium 24h: 23.303±3.632 x10^3; CRBM-1990 48h: 49.679±4.469 x10^3 *vs*. non-conditioned medium 48h: 39.425±4.808 x10^3; CRBM-1990 72h: 107.288±13.510 x10^3 *vs.* non-conditioned medium 72h: 75.046±10.476 x10^3; CRBM-1990 96h: 150.053±20.507 x10^3 *vs.* non-conditioned medium 96h: 97.650±18.474 x10^3). Caki-1-conditioned medium induced a faster growth rate compared to the non-conditioned medium (24h: 20.425±1.075 x10^3; 48h: 58.325±1.525 x10^3; 72h: 119.450±11.450 x10^3; 96h: 214.937±40.437 x10^3). ACHN-conditioned medium induced a growth rate similar to CRBM-1990 (24h: 21.858±4.210 x10^3; 48h: 49.587±5.407 x10^3; 72h: 106.515±11.224 x10^3; 96h: 162.847±31.330 x10^3) (Figure 5).

Discussion

This study was aimed at comparing osteolytic cytokine expression and angiogenetic activity among two established renal carcinoma cell lines (ACHN and Caki-1) and a cell line newly isolated from a renal carcinoma bone metastasis. The use of cell lines isolated from metastases enables the assessment of peculiar aspects of the metastatic localization of the tumor. With regards to bone metastases of renal carcinoma, the biggest problems are caused by osteolysis, which brings about pathological fractures and vertebral collapse and neovascularization, that promotes the development of metastases and can lead to problems during surgical intervention.

Histologically, the metastasis was osteolytic, with an abundance of newly formed vessels. The cellular morphology of the CRBM-1990 line was typical of neoplastic cells, with numerous mitoses, varying-sized nuclei and rare apoptosis. To evaluate the tumorigenicity of these cells, we tested the ability to grow in anchorage-independent conditions by a soft-agar assay; the CRBM cell line, seeded at very low density, was able to form colonies in a semi-solid medium (data not shown). The ability of tumor cells to grow in the absence of contact with extracellular matrix should not be considered as an artefact of cultured cells: anchorage-independence correlates quite well with tumorigenicity and it can be considered the property that allows tumor cells to infiltrate surrounding tissues and to establish distal metastases (3).

To further characterize this renal carcinoma cell line and to confirm the primitive tissue origin of these tumor cells, we investigated MET expression. In fact, various genetic changes are involved in human renal cell carcinomas and it has been reported that a number of genetic alterations and activation of proto-onco-genes or inactivation of tumor suppressor genes are involved in its pathogenesis; they concern, for instance, the *MET* proto-oncogene product (MET), or epidermal growth factor receptor (EGFR) and transforming growth factor β receptor II (TGFβR). In particular, a close correlation exists between the over-expression of *MET* and the development of

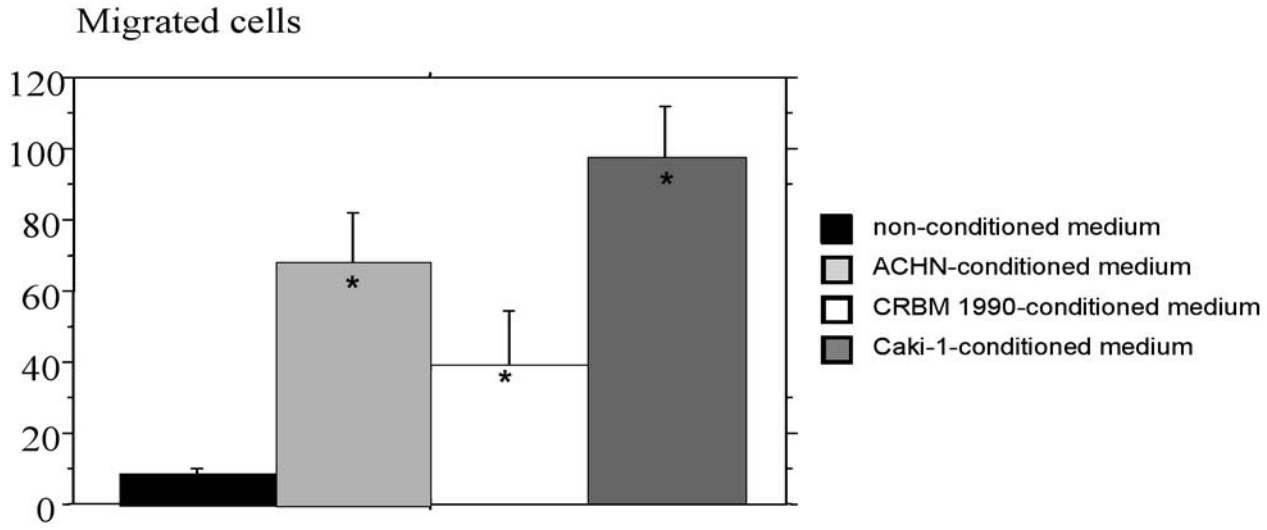

Figure 4. *Mean and standard error of the number of migrated BBE cells.*

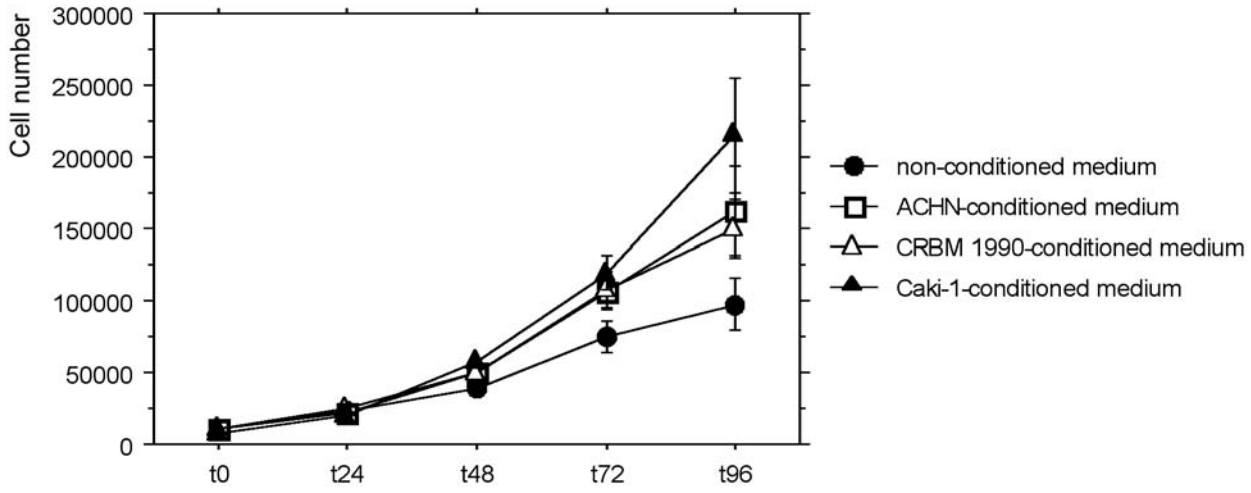

Figure 5. *Growth of bovine bone endothelial cells incubated with the CRBM-1990-, Caki-1- or ACHN-conditioned media.*

the chromophilic subtype of renal cell carcinoma with papillary growth pattern. HGF and its receptor, the MET, have varied biological functions in different tissues and have been implicated in mitogenic, motogenic and morphogenic responses and tumor suppression in tissue or organ regeneration and carcinogenesis (4). The renal carcinoma cell line characterized in this study expressed a high level of MET tyrosine-kinase receptor, like a well-known commercially available renal cell carcinoma model, the ACHN cell line, obtained from a primitive tumor.

Osteolytic bone resorption is mediated by factors stimulating the maturation and activation of osteoclasts. CRBM-1990, ACHN and Caki-1 cells expressed IL-6-specific mRNA to a similar extent, as was demonstrated for other metastatic renal cell carcinomas (5). However, RANK-L was not expressed, which is currently considered to be one of the most important factors for the activation of osteoclasts (6). This factor is produced by various neoplasms that are capable of producing osteolytic metastases, such as some neuroblastoma lines (Granchi D *et al. In vitro* blockade of receptor activator of nuclear factor-kappa B ligand prevents osteoclastogenesis induced by neuroblastoma cells. Int J Cancer, in press). Probably, not all bone metastases could induce osteolysis by the same mechanism, but there are peculiarities typical of different tumors. Osteolysis activation induced by renal carcinoma

could be mediated by RANK-L expression in the endothelial cells of the neoformed vessels. In turn, endothelial RANK-L could be induced by TGF-β which is highly expressed by CRBM.

TGF-β-specific mRNA was highly expressed by Caki-1 and CRBM-1990. TGF-β enhanced multinucleated osteoclast-like cell formation, the expression of TRAP and c-src induced by RANK-L (7). In addition, TGF-β up-regulated the expression of RANK-L in bone marrow-derived endothelial cells and in vascular endothelial cells, but not in osteoblasts (8).

The expression of mRNA specific for different forms of the pro-angiogenic factors VEGF, FGF-2 and also TGF-β was similar for Caki-1, CRBM-1990 and ACHN. VEGF-D and PlGF were not expressed by any of the three lines. As endothelium has been reported to have different properties according to different parts of the body (9), a bone endothelial line isolated from fetal bovine sternum (10) was used in the angiogenesis assays. Chemotaxis was assessed, because the first stimulus for neoangiogenesis is represented by a recruitment of endothelial cells to "colonize" the tumor. CRBM-1990-conditioned medium, such as Caki-1- and ACHN-conditioned media, induced the migration of endothelial cells. With regards to the stimulation of endothelial cell proliferation, the effect of CRBM-1990 was similar to that of ACHN and slightly less than that of Caki-1 cells. Therefore, there were no differences in the angiogenetic effect among the cells from the bone metastasis and the two established lines.

In conclusion, CRBM-1990 and ACHN cells had high levels of MET, that was constitutively expressed in the absence of ligand. IL-6 mRNA, that was involved in bone resorption and osteoclast activation, was expressed by Caki-1 and, at a lower level, by ACHN and CRBM-1990. TGF-β was expressed at a higher level by Caki-1 and CRBM-1990, and at a lower level by ACHN. However, the differences in cytokine mRNA expression were not significant. CRBM-1990 and ACHN did not express RANK-L. The three cell lines expressed angiogenetic growth factors and induced migration and proliferation of bone endothelial cells.

Acknowledgements

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References

1 Groessner-Schreiber B, Neubert A, Muller WD, Hopp M, Griepentrog M and Lange KP: Fibroblast growth on surface-modified dental implants: An *in vitro* study. J Biomed Mater Res *64A*: 591-599, 2003.

2 Streeten EA, Ornberg R, Curcio F, Sakaguchi K, Marx S, Aurbach GD and Brandi ML: Cloned endothelial cells from fetal bovine bone. Proc Natl Acad Sci USA *86*: 916-920, 1989.

3 Aaronson TA and Todaro GJ: Basis for the acquisition of malignant potential by mouse cells cultivated *in vitro*. Science *162*: 1024-1026, 1968.

4 Inoue K, Karashima T, Chikazawa M, Iiyama T, Yoshikawa C, Furihata M *et al*: Overexpression of c-met proto-oncogene associated with chromophilic renal cell carcinoma with papillary growth. Virchows Arch *433*: 511-515, 1998.

5 Paule B, Clerc D, Rudant C, Coulombel C, Bonhomme-Faivre L, Quillard J and Bisson M: Enhanced expression of interleukin-6 in bone and serum of metastatic renal cell carcinoma. Hum Pathol *29*: 421-424, 1998.

6 Shui C, Riggs BL and Khosla S: The immunosuppressant rapamycin, alone or with transforming growth factor-β, enhances osteoclast differentiation of RAW264.7 monocyte-macrophage cells in the presence of RANK-ligand. Calcif Tissue Int *71*: 437-443, 2002.

7 Koseki T, Gao Y, Okahashi N, Murase Y, Tsujisawa T, Sato T, Yamato K and Nishihara T: Role of TGF-β family in osteoclastogenesis induced by RANKL. Cell Signal *14*: 31-36, 2002.

8 Ishida A, Fujita N, Kitazawa R and Tsuruo T: Transforming growth factor-β induces expression of receptor activator of NF-kB ligand in vascular endothelial cells derived from bone. J Biol Chem *277*: 26217-26224, 2002.

9 Gori F, Hofbauer LC, Dunstan CR, Spelsberg TC, Khosla S and Riggs BL: The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. Endocrinology *141*: 4768-4776, 2000.

10 Formigli P, Orlandini SZ, Benvenuti S, Masi L, Pinto A, Gattei V, Bernabei PA, Robey PG, Collin-Osdoby P and Brandi ML: *In vitro* structural and functional relationships between preosteoclastic and bone endothelial cells: a juxtacrine model for migration and adhesion of osteoclast precursors. J Cell Physiol *162*: 199-212, 1995.

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