Abstract. Background: Osteoprotegerin (OPG) expression participates in the pathophysiology of osteoblastic metastasis in prostate cancer. Materials and Methods: We investigated whether the expression of OPG of PC-3 prostate cancer cells grown in 3-D collagen gels is stimulated by co-culture with MG-63 osteoblast-like cells. The expression of Runx2 (Cbfa1) and OPG were assessed by reverse transcription-polymerase chain reaction and Western blot analysis. Results: OPG and Runx2 were expressed in both PC-3 and MG-63 cells. OPG expression was remarkably enhanced in PC-3 cells grown in co-culture with MG-63 cells in a time-dependent manner. Runx2 expression of PC-3 cells was not altered by their co-culture with MG-63 cells. OPG expression of PC-3 cells was altered neither by insulin-like growth factor I (IGF-1), transforming growth factor β1 (TGFβ1), interleukin 6 (IL-6) nor by dexamethasone and zoledronic acid exogenously added to PC-3 cells. Conclusion: The enhancement of the OPG expression in PC-3 cells by MG-63 cells is not mediated by IGF-1, IL-6 and TGFβ1.

Advanced prostate cancer spreads almost always to the bones being responsible for the high morbidity and mortality of prostate cancer patients (1-3). Interestingly, metastasis of prostate cancer in bone produces osteoblastic lesions, characterized by an increased synthesis of woven bone (1-5). However, the initial phase of bone metastasis requires active bone resorption (1-3).

Osteoblasts express the receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) while its receptor RANK is expressed on circulating progenitor osteoclasts (6). The binding of RANKL to RANK stimulates osteoclastogenesis and marks the initiation site of bone remodeling (6-8). Moreover, osteoblasts secrete osteoprotegerin (OPG), which acts as a decoy receptor for RANKL, thus inhibiting osteoclastogenesis (9, 10). Furthermore, there is strong evidence that OPG, RANKL and RANK are involved in the pathophysiology of osteoblastic metastasis caused by prostate cancer (11-14).

Three-dimensional (3-D) type I collagen cell culture systems have been used for studying the biology of endothelial cells, endometrial cells, hepatocytes, osteoblasts, fibroblasts and various cancer cell lines in vitro inducing complex processes, such as angiogenesis, wound healing, functional and architectural integrity of various tissues under physiological and pathophysiological conditions (15-18). In particular, the 3-D collagen system has been proven useful in vitro models for analyzing the cell cell interactions between osteoblast-like cells and cancer cells in the context of bone metastasis, including that from prostate cancer (19-23). Moreover, several studies have suggested that a mechanism which involves an epithelial to mesenchymal transition of gene expression, facilitating tumour invasion and metastasis of tumours, includes the OPG/RANK/RANKL system (24-26).

Based on these data, we have used a 3-D collagen co-culture system to study the expression of osteoblast-related genes in PC-3 prostate cancer cells co-cultured with MG-63 osteoblast-like osteosarcoma cells. Therefore, we analysed the expression of OPG and Runx2 (Cbfa1), the latter being an osteoblast-specific transcription factor, in PC-3 prostate cancer cells co-cultured with MG-63 osteoblast-like cells.

Key Words: Osteoprotegerin, 3-D collagen, MG-63 osteoblasts, PC-3 cells.
Materials and Methods

Cell culture. PC-3 cells, an androgen-insensitive, p53-negative and Kirsten-Ras (K-Ras) mutated human prostate cancer cell line and the human MG-63 osteoblast-like osteosarcoma cell line were obtained from the American Type Cell Culture (ATCC, Bethesda, MD, USA). Cells were grown in 75 cm² culture flasks at 37°C in 5% CO₂ using Dulbecco’s modified Eagle’s medium (DMEM; Cambrex, Walkerville, MD, USA) containing 10% fetal bovine serum (FBS; Cambrex), 100 μg/ml, 100 mg/ml penicillin/ streptomycin (Cambrex), 1% L-glutamine (Cambrex) and 1% Hepes (Cambrex). The PC-3 and the MG-63 cells were grown to confluence and then detached by trypsin-EDTA solution and collected by centrifugation.

Preparation of the 3-D collagen gel co-culture system. Collagens were prepared using native type I collagen extracted from rat-tail tendons. Three-dimensional gels of native type I collagen were prepared in 24-well plates (6 each time) by rapidly mixing cells (at a final density of 10⁶ cells/ml of gel) with type I collagen in a mixture of fivefold concentrated minimum essential medium (MEM)-sodium hydroxide (0.1 M), and sodium bicarbonate (0.26 M) as previously reported (23-24). Co-cultures of PC-3 and MG-63 cells were created by putting gels containing PC-3 cells (6 gels, 10⁶ cells/gel) in flasks containing MG-63 cells (about 6×10⁶ cells; final ratio 1:1) and vice versa as well as collagen gels containing PC-3 and MG-63 in empty flasks (19-23). The cell culture conditions in the co-culture system were the same as described above.

Cell treatments. In addition, we have assessed the effects of transforming growth factor β1 (TGFβ1), insulin-like growth factor I (IGF-1) and interleukin 6 (IL-6) on runx2 and opg expression in PC-3 cells. The selected doses [TGFβ1 (50 ng/ml; 48 h), IL-6 (50 ng/ml; 48 h), IGF-1 (50 ng/ml; 48 h)], which has been previously proven to be maximally effective in a PC-3 cell proliferation assay (27-29), were assessed for induction of runx2 and opg expression in PC-3 cells. In addition, in our study we assessed the effects of zoledronic acid (50 μM; 48 h) and of dexamethasone (100 nM; 48h), therapeutic agents used in the treatment of advanced prostate cancer, on the expression of runx2 and opg in PC-3 cells (27).

RNA isolation and RT-PCR. Trizol Reagent (Invitrogen Corp., Carlsbad, CA, USA), SuperScript II RNase H- Reverse Transcriptase (Invitrogen), 100 mM dNTP Set (Invitrogen), Random Primers (Invitrogen), Marker 100 bp DNA Ladder (Invitrogen), Taq DNA Polymerase recombinant (Invitrogen), Hot Start Taq DNA Polymerase kit (Qiagen, Hilden, Germany) were used for RNA isolation and RT-PCR. Total RNA from MG-63 and PC-3 cells was extracted using the Trizol Reagent. First-strand cDNA was synthesized from 2 μg of RNA mixed with 0.5 mM Primer: Competimer ratios were 1:9 mixture and 1 μl cDNA. The Primer: Competimer was set at 1:9 for OPG and 1:9 for Runx2. After amplification, PCR samples were run in 1.8% agarose gels and the products were visualized by ethidium bromide staining and photographed by a Kodak DC290 camera. The quantifications of PCR products were performed with Kodak EDAS 290 program (27).

Protein isolation and Western blot. Tris-HCl (Sigma, St. Lious, MO, USA), EDTA (Sigma), dichlorodiphenyldichloroethane (DTT) (Sigma), NaCl (Sigma), leupeptin (Sigma), pepstatin (Sigma), aprotinin (Sigma), phenylmethanesulphonylfluoride (PMSF) (Sigma), NaF (Sigma), Na₃VO₄ (Sigma), nitrocellulose membrane (BIO-RAD Laboratories, Hercules, CA, USA) were used for protein isolation and Western blot analysis. After the co-cultivation, the cells were detached using trypsin-EDTA and then collected by centrifugation. The pellet was then lysed in a lysis buffer consisted of 20 mM Tris-HCl, pH-7.4, 1% TritonR X-100, 1 mM EDTA, 5 mM DTT and 150 mM NaCl, supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM NaF and 1 mM Na₃VO₄. This solution was left on ice for 20 min and recentrifuged. The protein content was determined using the Bradford protein assay. A total of 10-20 μg of total protein was resolved by 10% SDS-PAGE for the detection of OPG and Runx2 proteins. The gels were transferred onto nitrocellulose transfer membranes and blocked with 5% non-fat dry milk for 1 hour at room temperature. The membranes were incubated overnight at 4°C, with OPG antibody (R&D Systems, Minneapolis, MN, USA) at a 1:500 dilution or Runx2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1000 dilution, in TBS-Tween containing 1% non-fat dry milk. The blots were then washed and incubated with the appropriate secondary antibodies for 1 hour at room temperature (Santa Cruz Biotechnology; 1:2000 dilution). Expression of GAPDH (Santa Cruz Biotechnology) as reference protein was also analysed in the same protein extracts. The bands were visualized by exposure to X-ray film after incubation of the blots with SuperSignal ECL substrate (Pierce Biotechnology, Rockford, IL, USA). Quantification was performed using Kodak imaging software (EDAS Kodak software) (27).

Statistical analysis. All experiments were performed in triplicate. Values are mean±standard deviation (SD). Statistical analysis was performed by Student’s t-test. The level of statistical significance was set at p<0.05.
Results

Expression of OPG and Runx2 in PC-3 and MG-63 cells. OPG and Runx2 were expressed in the PC-3 prostate cancer cell line both at the mRNA (Figure 1A) and at the protein level (Figure 1B). These data suggest that PC-3 (human bone metastasis-derived prostate cancer cells) expressed genes, which have been strongly related with osteoblast/mesenchymal cell phenotype. OPG and Runx2 expression was also detected in MG-63 cells, as expected (data not shown).

The expression of OPG was significantly increased in 3-D collagen gels, containing PC-3 cells, co-cultured in flasks with MG-63 osteoblast-like cells at both mRNA (Figure 2A) and at protein level (Figure 2B). This was a time-dependent effect. However, we detected no significant changes in Runx2 expression in PC-3 cells co-cultured with MG-63 cells, neither at the mRNA (Figure 3A) nor at the protein level (Figure 3B). These data suggest that the enhancement of OPG expression was gene specific in this co-culture experiment.

In addition, IGF-1, TGFβ1 and IL-6 (50 ng/ml final concentration) did not significantly alter OPG (Figure 4A) or Runx2 expression (Figure 4B) in PC-3 cells. Nor did, zoledronic acid (50 μM) or dexamethasone (100 nM) affect the baseline OPG (Figure 5A) and Runx2 expression (Figure 5B) of PC-3 cells. These data suggested that enhancement of OPG expression in PC-3 cells induced by MG-63 cells was not mediated by IGF-1, TGFβ1 or IL-6 production of MG-63 cells.

Discussion

More than 90% of patients with advanced prostate cancer finally develop skeletal lesions (1-4). The pathogenesis of bone metastasis involves specific host-tissue recognition of circulating prostate cancer cells, enabling tumour cells to migrate and to invade the bone matrix and, finally, to establish local cell–cell interactions with host tissue, leading to osteoblastic metastasis (1-5). Notably, osteoblastic metastasis represents the stronghold of prostate cancer progression towards the androgen ablation-refractory stage.
However, the predominantly bone-specific nature of the refractoriness to androgen ablation therapy, implies that specifically local environmental cues, and not only genetic factors related to clonal evolution of the tumour, might be responsible for rescuing prostate cancer cells from apoptosis induced by androgen deprivation (30-33). It might be that host tissues such as bone that are rich in IGF-1, TGFβ1 and IL-6 are sanctuaries for prostate cancer cells, and this possibility might also account for the change of prostate cancer cells from an androgen-dependent to an androgen-independent phenotype while they still express active androgen receptors (5, 27-31). Therefore, cell cell interactions, involving IGF-1, IL-6 and TGFβ1, between prostate cancer cells and host tissue (osteoblasts) determine the host tissue reaction (blastic reaction) and tumour cell survival in bone metastasis microenvironment.

Recently, it was found that osteoblasts cells express a member of the tumor necrosis factor (TNF)-ligand family, RANKL, which is a membrane associated factor. RANKL interacts with OPG, a soluble decoy receptor for RANKL, preventing the establishment of osteolytic lesions in bone, although it did not prevent subcutaneous tumour growth, in vivo (34). Herein we documented that the presence of MG-63 osteoblast-like cells in our 3-D collagen culture system remarkably stimulated the expression of OPG in PC-3 prostate cancer cells. This enhancement of OPG expression was gene specific because Runx2 expression was not altered by this experimental procedure. Runx2 (Cbfa1) is a member of the runt homology domain family of transcription factors, essential for osteoblast differentiation and bone formation (35). It has been shown that Runx2 regulates the expression of all the major genes expressed by osteoblasts, related to mesenchymal/osteoblast phenotype. Runx2 expression is up-regulated when osteoblasts are treated in vitro with conditioned medium obtained from prostate cancer cells (36).

However, in our study Runx2 expression was neither enhanced by the co-culture of PC-3 cells with MG-63 cells nor by IGF-1, IL-6 and TGFβ1. Furthermore, the mediator(s) of such an enhancement of OPG expression of PC-3 cells induced by MG-63 cells was apparently not related to osteoblast-derived IGF-1, IL-6 and TGFβ1. Moreover,
zoledronic acid and dexamethasone, two pharmaceutical agents used in advanced prostate cancer treatment, did not modify OPG expression of PC-3 cells. Our data are in accordance with previous studies which have shown that OPG overexpression decreases osteolytic lesions although it does not affect the proliferation of cancer cells (34-36).

Therefore, the enhanced secretion of OPG by prostate cancer cells after their establishment in bone can influence the local cell cell interactions, reducing osteoclastogenesis locally and possibly provides the basis for the development of the blastic reaction in bone metastasis from prostate cancer (9-11). Notably, OPG also facilitates the survival of prostate cancer cells in vitro and this anti-apoptotic property is related to its ability to bind to and inhibit the TNF-related apoptosis-inducing ligand (TRAIL) death-activating receptors (9, 34). These findings suggest that bone marrow stromal cells express and secrete OPG in their microenvironment and that of prostate cancer cells, thus increasing the survival capacity of the latter. This OPG overproduction along with other bone-related survival factors, such as IGF-1, IL-6 and TGFβ1, could mediate the development of androgen ablation refractoriness and chemotherapy resistance of advanced prostate cancer (27-29).

In addition, the expression of Runx2 and OPG genes by prostate cancer cells, as documented in our study, supports the mesenchymal transition of prostate cancer cells during the metastatic process, as previously suggested (13, 14). Interestingly, bone microenvironment-related factors, such as IGF-1, TGFβ1 and IL-6, as well drugs used in the treatment of prostate cancer patients with bone involvement, such as zoledronic acid and dexamethasone, showed no effect on the expression of Runx2 and OPG in PC-3 cells. These data suggest that the epithelial to mesenchymal transition of prostate cancer cells during the process of invasion and metastasis is possibly not dependent on such growth factors. In conclusion, enhancement of OPG expression of PC-3 cells when co-cultured with osteoblast-like cells suggests that OPG may be a key player in the development of blastic reaction in the bone metastasis microenvironment.

References


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