Arachidonic acid modulates the crosstalk between prostate carcinoma and bone stromal cells

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Abstract

Diet high in n-6 fatty acids are associated with an increased risk of bone metastasis from prostate carcinoma (PCa). The molecular mechanism underlying this phenomenon is largely unknown. Arachidonic acid (AA) and its precursor linoleic acid can be metabolized to produce pro-inflammatory cytokines that act as autocrine and paracrine regulators of cancer behavior. We and other authors have previously reported that factors released by PCa cells excite an aberrant response in bone marrow stromal cells (BMSCs). We planned to study how AA may modulate in vitro the interaction between PCa cells and human BMSCs. First, we observed that AA is a potent mitogenic factor for PCa cells through the production of both 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) metabolites. While 5-LOX controls cell survival through the regulation of the Bcl-2/Bax ratio, COX-2 activity stimulates the release of transforming growth factor-α (TGF-α) and pro-inflammatory cytokines. The blockade of COX-2 activity through a specific inhibitor is sufficient to repress AA-induced gene transcription. The over-expression of transforming growth factor-α (TGF-α), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by AA-primed PCa cells resulted particularly effective in modifying cell behavior of cultured human BMSCs. In fact, we observed an increment in the cell number of BMSCs, due prevalently to the action of TGF-α, the number of osteoblasts, and the production of receptor activator for nuclear factor κ B ligand (RANKL), events mainly controlled by inflammatory cytokines. These findings provide a possible molecular mechanism by which dietary n-6 fatty acids accumulating in bone marrow may influence the formation of PCa-derived metastatic lesions and indicate new molecular targets for the therapy of metastatic PCa.

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Introduction

Increasing evidence associates dietary behavior to cancer. One of the most consistent findings in this matter suggests that long-chain polyunsaturated fatty acids may play an important role in cancer development and progression. In particular, epidemiological and animal model studies have indicated that the ratio of long chain n-6 to n-3 fatty acids may be associated with the risk of cancer (Terry et al. 2003). Arachidonic acid (AA) and its precursor, linoleic acid, can directly stimulate in vitro growth of several types of cancer cells, including human prostate cancer (PCa) cells. Moreover, a strong association between n-6 fatty acids content in the diet and the risk of metastatic PCAs exists (Wynder et al. 1994, Wang et al. 1995, Rose 1997).

AA is present in cell membranes as phospholipids, and it is released by cytosolic phospholipase A2 in response to a number of stimuli, including activation of membrane-bound receptors. However, a strict relationship between the amount of available AA in the membrane and the diet exists. In fact, as demonstrated by preclinical studies, intake of dietary n-6 fatty acid regulates the levels of AA not only in serum but also in membrane phospholipids (Kobayashi et al. 2006). AA is metabolized via either the cyclooxygenase (COX) or lipoxygenase (LOX) pathway producing prostaglandins...
and hydroperoxyeicosatetraenoic acids (HETEs) respectively. The development of tumor in humans and experimental animals is consistently linked to aberrant AA metabolism through the COX and LOX pathways (Furstenberger et al. 2006). Aberrant AA metabolism in tumor cells seems to be dependent on the action of COX-2 and specific LOX (Cuendet & Pezzuto 2000), and these enzymes are over-expressed in PCa in a strict association with tumor grade (Madaan et al. 2000). Although different LOXs may exert opposite effects on PCa cells, recent studies have focused on 5-LOX activity as a key regulator of tumor aggressiveness in PCa cell lines (Ghosh & Myers 1998a, Moretti et al. 2004, Hassan & Carraway 2006). Moreover, 5-LOX has been considered an attractive target, together with COX-2, in antitumoral therapy, in cancer types possessing a basal high capacity in metabolizing AA, like PCa (Gugliucci et al. 2002).

Most patients with advanced PCa develop bone metastases and suffer from long-term skeletal morbidity, involving pain, pathological fractures, and spinal cord compression, with a very significant impact on the quality of life. The preferential spreading of PCa cells to bone may be explained by the fact that bone marrow represents a favorable microenvironment for the growth of cancer cells with a specific genotype. At the same time, PCa cells mimic in some instances bone cells perturbing the normal process of bone remodeling and providing a niche in which they can grow and expand. Clinical evidence of bone metastasis from PCa demonstrates the existence of an intense crosstalk between cancer and bone cells resulting in mixed bone lesions controlled by an aberrant activation of osteoblasts and osteoclasts (Vessella & Corey 2006). Thus, the molecular interaction between bone and tumor cells appears to be a critical step in the growth of metastatic cells in the bone. To date, an effective anti-metastatic targeted therapy for Pca has not been found, and new data able to clarify the underlying molecular mechanisms are needed.

In this report, we analyze the effect of exogenous AA on metastatic PCa cells. In particular, we evaluate in vitro the possible role of AA in determining the formation of metastasis in bone microenvironment. Our data support a dual pro-metastatic effect of an AA-enriched environment: 1) assuring a high proliferative rate in tumor cells and 2) determining through the tumor metabolism an abnormal response in bone stromal cells. In vivo evidence supporting this hypothesis and justifying new therapeutic strategies is needed.

### Materials and methods

#### Cell culture

Human prostate cancer cell lines, PC3 and LNCaP, and breast cancer cell lines, MDA-MB-231 and MCF-7, were originally obtained by ATCC (Rockville, MD, USA) and maintained in DMEM (except LNCaP cells cultured in RPMI) supplemented with 10% fetal bovine serum, glutamine, and penicillin–streptomycin (Sigma). Human stromal cells were isolated from surgical human bone specimens obtained by routine clinical practice with the informed consent of the patients as described previously (Ciapetti et al. 2006). For the evaluation of osteoblasts content, bone stromal cells were fixed in 4% formaldehyde in 0.1 phosphate buffer, pH 7.2, and then incubated for 30 min with a solution of 2% Fast violet B salt (Sigma) and 4% Naphthol AS-MX phosphate alkaline solution (Sigma). In order to evaluate cell proliferation, cells were plated at a density of 10⁴ cells/cm², at various days of culture, and were recovered with a solution of trypsin/EDTA; washed twice with ice-cold PBS; and counted in a Burker slide. Conditioned medium (CM) was obtained culturing sub-confluent cells for 48 h in a medium without serum. At the end of incubation, the CM was recovered and centrifuged. The amount of residual AA in the CM was evaluated in a pivotal experiment using tritiated AA and measuring the radioactivity in the culture supernatant. After 48 h of culture, the residual AA was lower than 10 nM.

#### Colony growth assay

We evaluated PC3 cells capacity for growth at clonal density, plating cells at densities of 10 cells/cm² in 10% fetal bovine serum-supplemented DMEM. After 2 weeks of culture, adherent cells were fixed with cold methanol for 10 min, washed with PBS/BSA, and air-dried for 5 min. Adherent cells were stained with 0.5% crystal violet for 15 min at room temperature. The stained colonies were photomicrographed and analyzed by number and size with the public domain software ImageJ (by Wayne Rasband, http://rsb.info.nih.gov/ij/).

#### Real-time PCR

Total RNA was extracted from the cultured cells using Genelute Mammalian Total RNA kit (Sigma), and 1 μg RNA was used to synthesize cDNA (SuperScript III Platinum Kit, Invitrogen). Real-time PCR analysis was performed using Stratagene MX3000P personal Q-PCR (Stratagene, La Jolla, CA, USA) and SuperScript III Platinum Kit.
For all genes, 5 μl cDNA were used except for GAPDH amplification that was performed using 2.5 μl cDNA. Primers were designed using the online tool Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and the sequences were as shown in Table 1. Mean threshold cycle (Ct) values were determined by Stratagene software using three distinct amplification curves for each gene. Relative expression of the target gene was estimated using the formula: relative expression = 2^−ΔCt, where ΔCt = Ct (target gene)−Ct (GAPDH).

Western blotting
Total cell lysates were obtained by resuspending the cells in a buffer containing 1% Triton, 0.1% SDS, 2 mM CaCl2, and 100 μg/ml phenylmethyl sulfonyl fluoride. Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratory). Eighty micrograms of proteins were electrophoresed in 10% SDS–polyacrylamide gel and then electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was incubated with 1 μg/ml primary antibody and then with specific horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using a chemiluminescent detection system (Amersham Biosciences). Primary and secondary antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA, USA) except blocking antibodies against tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) which were purchased from Calbiochem (Darmstadt, Germany).

Statistical analysis
Results are expressed as means ± s.d. for at least three distinct experiments. Demonstration of significant differences among means was performed by Student’s t analysis considering 0.01 as the threshold value of P. All statistical analyses were performed using Kaleidagraph 3.6 (Synergy Software, Reading, PA, USA).

Table 1 Sequences of primers used in the real-time PCR amplifications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tr>
<td>EGF</td>
<td>5′-TCACCTCAGGGAAAGATGACC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-CAGTCCCACCCTCAGGT-3′</td>
</tr>
<tr>
<td>TGF-α</td>
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<td></td>
<td>5′-AAGCTCGTACACACCTCAG-3′</td>
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<td>TNF-α</td>
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<td></td>
<td>5′-AGGCCCAAGTTGAAATCTT-3′</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5′-GGGCCCTCAAGGAAAGAATC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-TTCTGGTGAAGGATGCTGTA-3′</td>
</tr>
<tr>
<td>RANKL</td>
<td>5′-CCTTGTGCTACTCTGACTATT-3′</td>
</tr>
<tr>
<td></td>
<td>5′-AATGTGGCATAAGGTAATAA-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GGCTCTGAAGGAGTGGACC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-AGGGGTTCATGGAAGCT-3′</td>
</tr>
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Figure 1 Effects of exogenous arachidonic acid on PC3 cells. (a) PC3 cells were treated for 48 (black bars) and 72 h (white bars) with increasing doses of arachidonic acid (μM), and the cell number was recorded and expressed as percentage with respect to control (100%). (b) PC3 cells were treated with 10 μM arachidonic acid (AA), an inhibitor of 5-lipoxygenase (MK886, 1 μM), an inhibitor of cyclooxygenase-2 (MK663, 10 μM), and combinations of these substances. Cell counts were recorded at 48 (black bars) and 72 h (white bars). (c) Protein expression detected by western blot of Bcl-2, Bax, and actin in the presence of arachidonic acid (AA, 10 and 50 μM) and AA plus 1 μM MK886. In the histograms on the right, relative densitometric values for each band are reported.
Results

Exogenous AA modulates PCa cells' proliferative status

A dose-dependent cell response to AA at 48 and 72 h was evaluated (Fig. 1a). AA determined a strong induction in cell proliferation evident from 48 h and lasting at least until 72 h. Treatments with concentrations higher than 10 μM AA were progressively cytotoxic. In order to analyze the metabolic pathways involved in AA-induced proliferation, we used two chemical specific inhibitors for 5-LOX (MK886) and COX-2 (MK663). MK663 selectively inhibits COX-2 with an IC50 value of about 1 μM for COX-2 and a selectivity ratio (COX-1/COX-2 IC50) for the inhibition of COX-2 of 106 (Riendeau et al. 2001). MK886 is a 5-LOX ‘translocation inhibitor’ with an IC50 value of 0.02 μM (Parkar et al. 1990). Since MK886 has also been demonstrated to decrease PC3 cell proliferation independently from 5-LOX inhibition, we used it at a concentration (1 μM) that did not have a significant cytotoxic effect on PC3 cell (Fig. 1b). 5-LOX inhibitor but not MK663 was able to annihil the

Figure 2 (a) Relative mRNA expression by q-PCR in PC3 cells treated with arachidonic acid. PC3 cells were incubated with arachidonic acid for 12 h alone or in the presence of the inhibitor of 5-lipoxygenase (MK886, 1 μM) or the inhibitor of cyclooxygenase-2 (MK663, 10 μM). Relative gene expression was calculated assigning the reference value of 1 to the untreated cells. Each value represents the mean (± S.D.) of three different experiments. (*P < 0.01 treated versus control). (b) Protein expression detected by western blot of COX-2, TNF-α and actin in the presence or absence of 10 μM arachidonic acid in total cell lysates of PC3, MDA-MB-231, LNCaP, and MCF-7 cells.

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proliferative effect of AA both at 48 and 72 h. When the protein expression of Bcl-2 and Bax is analyzed, we observed a thigh linkage between their relative presence and the concentration of AA (Fig. 1c). While 10 μM AA determined an anti-apoptotic equilibrium, upregulating Bcl-2 and downregulating Bax, 50 μM AA had an opposite effect, stimulating a pro-apoptotic status. Moreover, MK886, but not MK663 (data not shown), counteracted the upregulation of Bcl-2 in the presence of AA (Fig. 1c).

Gene transcription induction by AA

PC3 cells were cultured in the presence of 10 μM AA for 12 h, and total mRNA was isolated and screened in real-time PCR with a panel of primers specific for inflammatory cytokines. AA stimulated a significant upregulation in TNF-α and IL-1β mRNA (Fig. 2a). In order to evaluate the existence of an autocrine effect based upon the secretion of growth factors, we evaluated the expression levels of mRNAs for epidermal growth factor receptor (EGFR) ligands, EGF and transforming growth factor-α (TGF-α). TGF-α gene transcription was stimulated by AA treatment with an increase of about twofold with respect to the value in the untreated cells (Fig. 2a). On the contrary, EGF mRNA did not demonstrate a significant variation with respect to control values. The concomitant addition of MK663, but not MK886, was able to block completely the gene transcription effect of AA. At the same time, the addition of the inhibitors did not change in a significant manner the basal values of expression of the examined genes. We analyzed the protein expression level of COX-2 and TNF-α in highly osteotropic PC3 and MDA-MB-231 cells and in low aggressive, non-metastatic LNCaP and MCF-7 cells (Fig. 2b). COX-2 was detectable in all the cell lysates but PC3 and MDA-MB-231 cells expressed it at the highest level. Only in PC3 cells AA stimulated after 24 h a detectable upregulation of COX-2. In basal culture conditions, TNF-α was evident only in PC3 cells. The addition of AA determined the upregulation of TNF-α, both in PC3 and MDA-MB-231 cells but not in other cell lines.

Autocrine effects of AA-induced TGF-α

The autocrine role of factors released upon AA treatment was evaluated using the CM from PC3 cells primed for 48 h with 10 μM AA. The CM was added to PC3 cells cultured in serum-free medium, and the cell number was evaluated after 72 h (Fig. 3a). The CM from AA-treated PC3 cells determined an increment of about 30% in the cell number with respect to untreated cells. When we used the CM from PC3 cells treated with AA and MK663, we did not observe any significant effect. The proliferative stimulus induced by the CM was blocked by EGFR inhibitor ZD1839, suggesting that TGF-α may play a leading role in the autocrine growth of PC3 cells. The same effects were detected at a greater extent when we treated PC3 cells cultured at low density (Fig. 3b). In this experimental situation, the CM from AA-primed PC3 cells determined a significant increase in the number and extension of visible PC3 cell colonies after 14 days. Blocking EGFR activation determined a significant reduction in the growth of cell colonies both in basal culture condition and in the presence of AA CM.
Paracrine effects on human BMSCs

We cultured stromal cells from human bone marrow (BMSCs) with the CM from AA-treated PC3 cells and evaluated BMSC cell number with respect to control after 72 h of culture (Fig. 4a). The CM from AA-treated PC3 cells determined an increase in the number of BMSCs of ~30% with respect to untreated BMSCs. The CM from AA + MK663-treated PC3 cells did not determine any significant difference with respect to control cells. The increase in proliferation was abolished by the addition of the inhibitor ZD1839, suggesting a leading role for EGFR transduction axis. At the same time, we evaluated the presence of osteoblasts through the detection of alkaline phosphatase (ALP) activity. In the BMSC population cultured in 15% fetal bovine serum-supplemented medium plus one-fourth of PC3 CM we detected <5% of ALP-positive cells. This percentage significantly grew in the presence of CM from AA-treated PC3 cells, but not with the CM from AA + MK663-treated PC3 cells, reaching 10% of the total BMSC population (Fig. 4b). As demonstrated by the addition of ZD1839, the formation of osteoblasts is only partially dependent on EGFR functioning (Fig. 4b).

The role of TNF-α and IL-1β secreted by PC3 cells on BMSCs

In order to verify the effect of TNF-α and IL-1β present in PC3 CM on osteoblast differentiation, we treated BMSCs with the CM from AA-treated PC3 cells in the presence of the blocking antibody against TNF-α or IL-1β. When the expression of ALP activity in BMSCs is analyzed, we observed that the antibody anti-TNF-α completely blocked the differentiative effect of AA CM, reverting the percentage of osteoblasts to expected values (Fig. 5a). On the contrary, the blocking antibody against IL-1β was not able to reduce significantly the differentiative effect of AA. As demonstrated in our previous data, PC3 CM is

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**Figure 4** The paracrine effect of arachidonic acid-primed PC3 on human bone stromal cells (BMSCs). (a) Conditioned media from PC3 cells untreated or treated with 10 μM AA (AA CM) or with AA plus 10 μM MK663 (AA + MK663 CM) for 12 h were added to BMSCs cultured at sub-confluent density. BMSCs were cultured also in the presence of AA CM plus 10 μM ZD1839. Cells were treated for 72 h and then counted. Three different experiments were performed and mean values (± s.d.) are reported. The images on the right show exemplificative optical fields for each experimental group as visualized by contrast phase microscopy. (b) Conditioned media from PC3 cells untreated or treated with 10 μM AA (AA CM) or AA plus 10 μM MK663 (AA + MK663 CM) for 12 h were added to BMSCs. The involvement of EGFR activation was tested culturing BMSCs in the presence of AA CM and 10 μM ZD1839. Cells were cultured for 72 h and then stained for the presence of alkaline phosphatase (ALP). Positive cells were counted and percentages with respect to the total number of BMSCs were calculated. Three different experiments were performed and mean values (± s.d.) are reported. The images on the right show exemplificative field for each experimental group. Dark cells are those positive for ALP activity. (*P < 0.01 treated versus control).
able to induce the receptor activator for nuclear factor κ B ligand (RANKL) expression in BMSCs (Angelucci et al. 2006). We observed that treating PC3 cells with AA determined the release of factors that are able to enhance the gene transcription of RANKL in BMSCs (Fig. 5b). The effect of AA CM was only partially counteracted by the EGFR inhibitor ZD1839, while the concomitant addition of antibody anti-TNF-α or anti-IL-1β determined a drastic reduction in RANKL mRNA expression with respect to AA CM-treated BMSCs (Fig. 5b).

Discussion

Several authors have previously demonstrated that tumor cells over-express AA metabolizing enzymes, and this feature may establish a bridge between the dietary fat intake and the progression of cancer. In particular, dietary n-6/n-3 fatty acid ratio seems to control not only the membrane fatty acid composition but also the growth of PCa xenografts in preclinical models (Kobayashi et al. 2006). Therefore, we may propose that PCa cells over-producing LOX and COX enzymes are able to take an important advantage for their growth from the presence of elevated concentrations of AA in their microenvironment. Our study and a plethora of data from other authors indicate that both LOX and COX pathways have to be considered important in the definition of AA-induced tumor aggressive behavior. The most evident effect of AA on PCa cells is a dose-dependent induction of cell proliferation. Products responsible for mitogenic effect of AA have included eicosanoids as well as several LOX metabolites. This mitogenesis is blocked if partial metabolism of AA is interrupted by the use of MK886, the inhibitor of 5-LOX (Ghosh & Myers 1998b). Moreover, the levels of 5-HETE, the metabolite produced by 5-LOX, are strictly correlated in PC3 cells with DNA synthesis (Hassan & Carraway 2006). Interestingly, the same authors indicated that 5-HETE is a downstream effector in EGFR signaling. Our data confirm that 5-LOX has a leading role in the control of PCa cell proliferation in the normal culture conditions; in fact the inhibitor MK886 is able to suppress the mitogenic effect induced by AA completely. This phenomenon has a molecular counterpart in the relative expression levels of Bcl-2, elevated in the presence of AA, and Bax, downregulated by AA. The addition of MK886, but not of the COX-2 inhibitor MK663, was able to restore the basal levels of Bcl-2, thereby controlling the proliferative capacity of cells.

Epidemiological data indicate that high-fat diets, especially those rich in saturated n-6 fatty acids, can
affect bone health, especially during development and in aged individuals. One of the proposed mechanisms involves the generation of active fatty acid metabolites in bone marrow stromal cells, which are able to direct the proliferation and differentiation of these cells (Nuttall et al. 1998). Interestingly, a direct correlation between reduced bone density and increase in marrow fat seems to exist, a condition frequently observed in advanced age (Burkhardt et al. 1987). This evidence suggests that fat intake may influence the metastatic tumor cell growth in bone marrow and, in particular, in aged individuals. In agreement with this hypothesis are data indicating that PCa cells interact with adipocytes in vitro with a resultant increase in their proliferation (Tokuda et al. 2003), and that they localize to lipid-rich regions in bone marrow metastases. In particular, Brown et al. (2006) have demonstrated that AA promotes invasion in PC3 cells and may represent one of the key molecules that direct the PCa cells to the bone marrow. If we consider that EGFR activation is implicated in PCa cell migration and invasion, some of the features indicated by Brown may be explained through the AA-induced autocrine release of TGF-\(\alpha\). EGFR activation may play a pleiotropic role in bone metastasis formation. In fact, as demonstrated previously, factors released under the control of EGFR activation are able to modulate bone remodeling through the induction of RANKL in human osteoblasts (Angelucci et al. 2006). This evidence together with the data presented here suggests that AA and EGFR may share downstream common molecular events mediated by the transcription of the same genes. Moreover, since several authors have demonstrated that AA is a secondary messenger released upon EGFR activation (Hassan & Carraway 2006), we may hypothesize the existence of an autocrine loop maintaining EGFR continuously activated.

The CM from AA-primed PC3 cells stimulates in vitro a multifaceted response in human BMSCs (Fig. 6). TGF-\(\alpha\) through the activation of EGFR promotes the proliferation of BMSCs, but its action is not sufficient to explain the other effects on BMSCs. On the contrary, TNF-\(\alpha\) and IL-1\(\beta\) play a central role in modulating the formation of osteoblasts and the production of RANKL. Because IL-1\(\beta\) was able to stimulate the production of RANKL but not the formation of osteoblasts, we hypothesize that the two phenomena are controlled by different molecular mechanisms. For this reason, the TNF-\(\alpha\)/IL-1\(\beta\) ratio in a complex contest in which several other factors, usually in a net of reciprocal interactions, can modulate bone remodeling (see (Yoneda & Hiraga 2005)), may establish the outcome of bone lesion, lytic, or sclerotic. TNF-\(\alpha\) is a multifunctional cytokine defined as a leading factor in chronic inflammatory osteolysis is able to stimulate osteoclastogenesis (Kitaura et al. 2004).
Both stromal cells and osteoclast precursors are TNF-α targets, but stromal cells seem to make greater contribution in TNF-α-induced osteoclastogenesis (Kitaura et al. 2005). In our hypothesis, the BMSCs are fundamental in determining bone remodeling directed by PCa cells. In fact, BMSCs are both able to release osteoclastogenic factors, i.e., RANKL, and able to differentiate in osteoblasts, thereby controlling potentially both resorption and deposition of mineralized bone matrix (Fig. 6). In agreement with this hypothesis is the evidence that bone metastasis, also in the presence of a predominant type of lesion, develops partially by TNF-alpha-induced osteoclastogenesis in vivo using human cells and simulating a microenvironment rich in AA, it is possible that results obtained through the use of murine experimental models may differ from ours (Gamradt et al. 2005).

The proposed model may contribute to elucidate the largely obscure role of AA in PCa cancer progression. A diet rich in n-6 fatty acids can significantly alter the timing and extent of clinical outcome in PCa bone metastasis modulating the interaction between PCa cells and BMSCs.

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References


