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Effect of NSAIDs on the aminopeptidase activity of cultured human osteoblasts

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ABSTRACT

Aminopeptidases (APs) are involved in various physiological and pathological processes. In tumor tissues the expression of APs, cyclooxygenase-2 and its metabolites are increased. The objective was to determine the effect of certain NSAIDs on the AP activity of osteoblasts. Primary cultures of osteoblast were treated with different concentrations of indomethacin, meloxicam, naproxen, nimesulide, and piroxicam. The AP activity was fluorimetrically determined using aminoacyl-β-naphthylamides (aa-βNAs) as substrates: Ala-βNA, Arg-βNA, Gly-βNA, Leu-βNA, Lys-βNA, Met-βNA, and Phe-βNA.

The five NSAIDs showed an inhibitory effect of AP activity against the study substrates depending on the dose tested. Meloxicam and piroxicam had the highest inhibitory effect on enzymatic activity, with an IC_{50} of around 70 μM. Our results suggest that the physiological alteration of osteoblasts in the presence of NSAIDs may be a consequence of AP inhibition, suggesting a potential clinical role for these drugs against cancer in combination with chemotherapeutic agents.

1. Introduction

Aminopeptidases (APs) are generally zinc metalloenzymes that catalyze the cleavage of amino acids near the N-terminus of polypeptides, facilitating hydrolysis of the peptide bond (Taylor, 1993). APs are localized in different tissues and cells of the organism at membrane or intracellular level; however, they can also be secreted as soluble factors (Gabrilovac et al., 2004; Hitzerd et al., 2014; Kido et al., 2003; Lowther and Matthews, 2002; Sanz, 2007). APs are essential for important physiological processes such as protein maturation, degradation of peptides, and cell-cycle control, and they actively participate in electrolytic balance, immune response, and tumor processes. The supply of cellular free amino-acids regulated by APs is critical for the survival and proliferation of cancer cells. Importantly, many cancer cells are dependent on specific amino-acids, whose depletion has a greater impact on these cells than on normal cells (Scott et al., 2000).

The main function of osteoblasts is bone formation and repair, although they have also been attributed with immune functions (Díaz-Rodríguez et al., 2009; Eriksen, 2010; García-Martínez et al., 2006; Neve et al., 2011; Stanley et al., 2006). APs expressed by the membrane of these cells have activity against different substrates (Ala-βNA, Arg-βNA, Gly-βNA, Leu-βNA, Lys-βNA, Met-βNA, and Phe-βNA). This activity is partially inhibited by puromycin or bestatin which are classic inhibitors of AP activity (Lucena et al., 2013).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for their anti-inflammatory, analgesic, and antipyretic effects, which are exerted by cyclooxygenase (COX) inhibition. At high doses, NSAIDs have evidenced antitumor activity in different cell populations, inhibiting their growth capacity and reducing cell

List of abbreviations: APs, aminopeptidases; NSAIDs, non-steroidal anti-inflammatory drugs; aa-βNAs, aminoacyl-β-naphthylamides; COX, cyclo-oxygenase; PBS, phosphate-buffered saline solution; FCS, fetal calf serum; EDTA, ethylene diamine tetra-acetic acid; APN, aminopeptidase N.

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migration. This last effect contributes to the inhibition of tumor cell metastasization (Axelsson et al., 2010; Sobolewski et al., 2010a). At therapeutic doses, these drugs can also have diverse effects on osteoblasts, inhibiting their proliferation and differentiation and modulating their antigenic profile or some of their immune functions, e.g., phagocytic capacity (De Luna-Bertos et al., 2014, 2013, 2012; Díaz-Rodríguez et al., 2012a, 2012b, 2010). These data have raised questions about the modulating effect of NSAIDs on the AP activity of human osteoblasts.

With this background, the objective of this study was to determine the effect of different doses of indomethacin, meloxicam, naproxen, nimesulide, and piroxicam on the AP activity of cultured human osteoblasts against different AP substrates.

2. Materials and methods

2.1. Tissues

This study included samples from human trabecular bone sections obtained from healthy volunteers during the scheduled surgical extraction of impacted third molar. Informed consent was obtained from each patient.

2.2. Isolation and culture of osteoblasts

A sample of trabecular bone section from each of three Caucasian patients aged 20–30 years (2 women and 1 man) was independently processed. Sections were washed thoroughly in phosphate-buffered saline solution (PBS, pH 7.4) to remove marrow and were then seeded onto culture dishes (Falcon Labware, Oxford, UK) in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL gentamicin (Braun Medical SA, Jaén, Spain), 2.5 μg/mL amphotericin B (Sigma, St Louis, MO, USA) and 20% fetal calf serum (FCS) (Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Confluent monolayers were obtained after 3–6 weeks. Three highly pure osteoblastic lines (one per patient) were obtained from these cultures. The cells were detached from the culture flask with a solution of 0.05% trypsin (Sigma, St Louis, MO, USA) and 0.02% ethylene diamine tetra-acetic acid (EDTA) (Sigma, St Louis, MO, USA), and were washed and suspended in complete culture medium with 10% FCS. The cells were characterized by their morphological, biochemical (alkaline phosphatase activity and osteocalcin secretion), and antigenic profile ($CD10^+$, $CD13^+$ and $CD44^+$) as described by Lucena et al. (Lucena et al., 2013).

2.3. Enzymatic analysis

Cultured human osteoblasts were treated with concentrations ranging from 62.5 to 500 μM of indomethacin, meloxicam, naproxen, nimesulide, and piroxicam, for 24 h; untreated cells served as controls.

Ala-AP, Leu-AP, Arg-AP, Phe-AP, Lys-AP, Gly-AP, and Met-AP, were fluorometrically measured using the following aminoacyl-β-naphthylamides (aa-βNAs) as substrates: Ala-βNA, Leu-βNA, Arg-βNA, Phe-βNA, Lys-βNA, Gly-βNA, and Met-βNA. A modification of Greenberg's method (Greenberg, 1962) was adopted; placing 2 mL of a 1.5×10^4 cell/mL cell suspension in 24-well culture plates and incubating them for 24 h at 37 °C in 5% CO_2 atmosphere. The culture supernatant was then removed and 1 mL of 100 μM substrate solution was added. The substrate was dissolved with dimethyl sulfoxide (p/v) and diluted with phosphate-buffered saline, pH 7.4. Four wells were used as controls, adding 1 mL PBS. Plates were incubated for 60 min at 37 °C. All reactions were stopped by adding 1.5 mL of acetate buffer (100 mM, pH 4.2). The amount of aa-βNA present was fluorometrically measured at 412 nm emission wavelength with excitation wavelength of 335 nm. The number of cells was determined by flow cytometry (Ortho Cytoron Absolute. Ortho Diagnostic Systems, Johnson & Johnson Co, Milan, Italy). Membrane-bound AP activity was expressed as pmol of aa-β NA hydrolyzed per min per 10^4 cells/mL. Fluorogenic assays were linear with respect to incubation time and cell number. Results were expressed as the percentage of enzymatic activity with respect to controls.

2.4. Statistical analysis

SPSS v 22.0 software (SPSS Inc., Chicago, IL) was used for the statistical analysis. Data were expressed as mean (±standard deviation) percentage activity in the presence of NSAIDs with respect to controls in the three human culture lines analyzed. All experiments were performed at least three times in each culture line. An ANOVA was performed to establish the statistical significance of differences.

3. Results

In general, the AP activity (Ala-AP, Leu-AP, Arg-AP, Phe-AP, Lys-AP, Gly-AP, and Met-AP) of cultured human osteoblasts was dose-dependently inhibited by the presence of indomethacin, meloxicam, naproxen, nimesulide, or piroxicam for all cell lines assayed.

Indomethacin inhibited this AP activity at all doses studied, with IC_{50} values ranging between 50 and 136 μM (Fig. 1). The highest inhibitory action of indomethacin was for Phe-βNa and Gly-βNa and the lowest was for Ala-βNa, Lys-βNa and Met-βNa.

Meloxicam dose-dependently inhibited this activity (Fig. 2). The inhibition curve fitted a negative exponential equation, tending towards total enzyme inhibition at the highest concentrations. The IC_{50} for all substrates was within a narrow range of around 70 μM. The highest inhibitory capacity was for Phe-βNA and Gly-βNA and the lowest for Arg-βNA, Lys-βNA, and Met-βNA (Fig. 2).

Naproxen weakly but significantly inhibited the AP activity of the human osteoblasts. Enzymatic activity was only reduced by <50% in the dose range studied, and no substantive differences in effects were found between the lowest and highest doses. At the highest doses the activity reached a plateau for all the substrates tested (Fig. 3).

Nimesulide inhibited the AP activity of the osteoblasts for all substrates in a dose-dependent manner (Fig. 4), with the exception of a plateau against the basic substrates (Arg-βNA and Lys-βNA) in the range of concentration 62.5–250 μM. However, the inhibitory effect of nimesulide ($IC_{50} \approx 150$ μM) was much higher than that of naproxen.

Piroxicam also produced a dose-dependent inhibition of AP activity for all substrates (Fig. 5); IC_{50} values were all within a narrow range of around 75 μM.

4. Discussion

The presence of indomethacin, meloxicam, nimesulide, or piroxicam, at doses of 62.5–500 μM, significantly inhibits the AP activity (Ala-AP, Leu-AP, Arg-AP, Phe-AP, Lys-AP, Gly-AP, and Met-AP) of cultured human osteoblasts. Naproxen showed inhibitory activity only at higher concentrations. Among these NSAIDs, indomethacin, meloxicam, and piroxicam showed greater AP inhibition for all substrates studied.

NSAIDs are frequently used in the treatment of bone lesions for their analgesic, antipyretic, and anti-inflammatory effects. Their inhibition of osteoblast AP activity may expand their potential