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The effect of heparin on osteoblast differentiation and activity in primary cultures of bovine aortic smooth muscle cells

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Abstract

Recent studies have suggested that aortic smooth muscle cells undergo a phenotypic transition into osteoblast-like cells and mineralize when cultured in the presence of β-glycerophosphate. Since we had previously demonstrated that heparin could inhibit osteoblast differentiation and mineralization in primary cultures of murine calvaria cells, we were interested in determining if heparin would have a similar effect when primary aortic smooth muscle cells were cultured in the presence of β-glycerophosphate. The effect of heparin and low molecular weight heparin (LMWH) on osteoblast differentiation and activity was therefore examined in primary cultures of bovine aortic smooth muscle cells (BASMC) over a 14-day period. Here, we report that BASMC differentiate into osteoblast-like cells when cultured in the presence of β-glycerophosphate. Moreover, we report that heparin not only inhibits this process but that it also inhibits the ability of BASMC to mineralize as well. Importantly, these effects were found not to be dependent upon heparins' anticoagulant activity since unfractionated heparin and heparins with low anti-thrombin III affinities inhibited the mineralization process equally well. Sulfation, however, was found to be a major determinant of heparins ability to inhibit BASMC mineralization since neither dermatan sulfate nor *N*-desulfated heparin were able to demonstrate an effect. We conclude that BASMC cultures can undergo a phenotypic transition into mature osteoblasts and that both the differentiation process and their ability to mineralize are inhibited by heparin.

Keywords: Anticoagulants; Heparin; Atherosclerosis; Muscle; Smooth; Calcium

1. Introduction

Calcification of the vessel wall can be a particularly troublesome complication of atherosclerosis [1]. It can increase vessel wall rigidity [2,3], augment plaque brittleness [4], and can lead to increased plaque rupture or vessel wall dissection following balloon angioplasty and/or stent placement [5–8]. Why vessel walls calcify is unknown. However, recent studies suggest that arterial calcification is not a passive event as was once believed. Rather, it appears to be a highly complex event that shares many similarities with the mineralization of bone. In support of this concept, several investigators have observed that primary cultures of vascular smooth muscle cells appear to undergo a phenotypic transition into osteoblast-like cells and mineralize in the presence of β-glycerophosphate [9–11]. In addition, recent studies have reportedly localized osteoblast specific markers to the calcified atherosclerotic lesions of human vessels [12,13]. When taken together, these findings suggest that osteoblasts may be responsible, under certain pathological conditions, for the calcification of arterial plaques.

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Numerous studies indicate that osteoblasts arise from mesenchymal stem cells that can differentiate along several lineages. In the bone marrow, mesenchymal stem cells differentiate not only into osteoblasts but also into adipocytes, chondroblasts, and smooth muscle cells [14–20]. Many factors are known to regulate the differentiation process including bone morphogenic proteins, interleukin-6 (IL-6)-type cytokines, Vitamin D3, and hormones such as parathyroid hormone. In addition, drugs such as dexamethasone and/or heparin are also known to modulate osteoblast differentiation and activity. Thus, we have shown that both osteoblast differentiation and activity are inhibited by heparin in primary cultures of murine calvaria cells [21] and that this can result in an inhibition of bone formation in vivo [22–24].

The purpose of the current study was two-fold. First, we wished to verify reports of primary smooth muscle cell cultures undergoing osteoblast differentiation and mineralization in the presence of β-glycerophosphate. In addition, we were also interested in determining if heparin could inhibit this process since we had previously demonstrated heparins' ability to inhibit osteoblast differentiation and mineralization in primary cultures of murine calvaria cells [21]. Here, we report that cultures of BASMC do undergo a phenotypic transition into mature osteoblast-like cells and that this process can be inhibited by heparin.

2. Materials and methods

2.1. Materials

Unfractionated heparin (173 anti-factor Xa U/mg) and the low molecular weight heparin (LMWH), Fragmin (100 anti-factor Xa U/mg), were a generous gift from Rhone-Poulenc Rorer, Montreal, Canada. Well-defined heparin fractions with molecular weights ranging from 3000 to 18000 Da were obtained from Enzyme Research Laboratories (South Bend, IN), whereas *N*-desulfated heparin was purchased from Sigma Chemical Co. (St. Louis, MO). Heparin with low affinity for antithrombin (LAH; anti-factor Xa activity < 1.0 U/mg) was produced by chemically modifying unfractionated heparin as described previously [24].

2.2. Primary bovine aortic smooth muscle cell isolation

Smooth muscle cells were harvested from fresh bovine aorta obtained from a local slaughter house as described previously [25]. Briefly, the aortas were denuded of endothelium and adventitia and the remaining medial tissue minced into 1–2 mm^3 pieces. The minced medial tissue was cultured for several weeks in DMEM (GIBCO BRL, Burlington, Ont.) containing 20% fetal bovine serum in order to allow for smooth muscle cell outgrowth from the cultured explants. Once confluency was reached, the cells were harvested and either used immediately or stored in liquid nitrogen. For all experiments, cells up to and including passage 3 were used. To confirm that the cells isolated from our explants were indeed vascular smooth muscle cells we stained the cells using a smooth muscle cell α-actin—specific antibody (Sigma Chemical Co., St. Louis, MO).

2.3. Differentiation assay

Bovine smooth muscle cells (5×10^4 cells/well) were cultured in 24 well plates in the presence or absence of 10 mM β-glycerophosphate using DMEM supplemented with15% fetal bovine serum and 0.5 mM ascorbic acid. In some experiments, increasing concentrations of heparin (0–50 μg/ml) or LMWH (0–50 μg/ml) were also included in the culture medium. Alternatively, heparin fractions of varying molecular weights, *N*-desulfated heparin, or low-affinity heparin (LAH), all at a concentration of 10 μg/ml, were used in place of heparin or LMWH. Media were changed every 3–4 days until the termination of the experiments. After 14 days, cells were fixed and the number of osteoblast-like cells quantified as described below.

2.4. Alkaline phosphatase staining and quantification

Osteoblasts were identified histochemically by alkaline phosphatase (ALP) staining as described previously [11]. Briefly, a diazonium salt solution was prepared by dissolving 12 mg of fast blue salt in 50 mL of 0.01% alkaline naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO). The cells were then fixed with 60% citrate-buffered acetone for 45 s and then immersed in the alkaline-dye mixture for approximately 30 min before being counter-stained with hematoxylin solution. Osteoblasts were identifiable by visible cytoplasmic staining of the precipitated azo dye at sites of ALP activity.

In some experiments, ALP was also quantified by using a commercially available assay (Sigma Chemical Co.). Briefly, the cells were washed three times with PBS, lysed with 1% Triton X-100, and then 2 μl of the cell lysate incubated with 200 μl of ALP reagent. Quantitative kinetic determination of cell associated ALP activity (U/mg) was then determined at 30 °C by monitoring absorbance at 405 nm on a Versamax microplate reader (Molecular Devices).

2.5. Quantitative RT-PCR analysis

BASMC were plated at a cell density of 2×10^6 /10-cm dish (Corning, NY) and then cultured to promote cell differentiation as described above. Fourteen days later, the cells were harvested and total RNA was isolated using an Rneasy RNA mini kit (Qiagen, Chatsworth, CA). For RT-PCR analysis, 4 μg total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Burlington, ON). PCR amplification reactions were performed with Supermix (Invitrogen, Burlington, ON) in the PTC-100 Programmable Thermal Controller (MJ Research Inc., Reno, NV). The specific primers and annealing temperatures for PCR are listed