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Low-Molecular-Weight Peptides in Bone Extracts that Stimulate Osteoblast Mitogenesis

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Summary: Although a majority of regulatory peptides elaborated by neuroendocrine cells are small, *i.e.*, less than 50-60 residues, no low-molecular-weight, bone-derived mitogenic peptides have been described. We have size-fractionated extracts of neonatal mouse calvaria, a rapidly forming bone, and assayed for osteoblast proliferation. Mitogenic peptides with estimated sizes of 1,600, 1,050, and 770 daltons were detected. Their protein nature was demonstrated by the reduction in mitogenic activity following protease treatment. Fibroblast mitogenesis was not stimulated by any of the peptides. These data indicate that there are mitogenic peptides in bone smaller than any previously described locally-derived bone cell growth factor.

A number of growth factors for the bone-forming osteoblast have been extracted from bone matrix or are secreted by the osteoblast (For Review, see 1). The smallest of these proteins are the 7,500-dalton IGF- I and II. Yet the majority of secretory proteins that regulate cell activities are smaller, with molecular weights of perhaps 6,000 or less (2,3). It is surprising, therefore, that no low-molecular-weight, bone-derived, bone-bioactive peptides have been identified. Brief mention, but no characterization, has been made of osteoblast mitogenic activities of 1,000 (4) and 3,600 (5) daltons. To test the hypothesis that there are small, locally-produced, bone cell growth factors, we utilized neonatal mouse calvaria, a rapidly developing bone. Mitogenic activity of calvarial extracts was assessed following gel filtration chromatography to resolve low-molecular weight peptides.

Materials and Methods

Extraction of calvaria

4-8-d-old neonatal Swiss Webster mice (Tyler Laboratories, Bellevue, WA) were sacrificed by cervical dislocation. The calvaria were removed, rinsed in phosphate-buffered saline, frozen in liquid nitrogen, and stored at -70°C until use. Approximately 500 calvaria were used for each extraction.

Abbreviations: IGF, insulin-like growth factor; VIP, vaswoactive intestinal peptide; α-MSH, CGRP, calcitonin gene-related peptide; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; and HEPES, N-2-hydroxyethylpiperazine-n'2-2ethanesulfonic acid.

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Calvaria were partially thawed, suspended in 4 vol of 10% acetic acid, and heated to 100°C for 10 min. After cooling the calvaria were disrupted with a Brinkmann polytron. The extract was centrifuged at 1,900 x *g* for 15 min. The supernatant was then centrifuged at 48,000 x *g* for 30 min. This high-speed supernatant was subjected to ultrafiltration first through a YM-10 filter, then a YM-5 filter (nominal 10,000- and 5,000- MW cutoff, respectively, Amicon, Danvers, MA). The low-molecular weight ultrafiltrate was taken to dryness in a Speed Vac concentrator (Savant, Farmingdale, NY).

The residue was resuspended in 10% acetic acid, centrifuged at 1,900 x *g* for 15 min and the supernatant removed and taken to dryness. This residue was suspended in 2 ml of 10% acetic acid, centrifuged, and the supernatant fractionated by gel filtration chromatography.

Gel filtration chromatography

The ultrafiltrate was fractionated on a column (1.1 x 91 cm) of Bio-Gel P-6, 200-400 mesh (Bio-Rad, Richmond, CA), equilibrated with 10% acetic acid containing 0.01% BSA. The column was calibrated with the following radioiodinated peptides: IGF-I (MW 7,650), ß-endorphin (MW 3,650), VIP (10-28)(MW 2,339), γ-endorphin (MW 1,860), α-melanotropin (MW 1,665), CGRP (28-37)(MW 1,182), and D-Tyr-Val-Gly (MW 337). Recovery of applied radioiodine was 75-85%. The flow rate was 5 ml/h and 2-ml fractions were collected. Aliquots of each fraction were dried in a Speed Vac concentrator. The residues were reconstituted in 1% acetic acid, except as noted below.

Preparation of osteoblast cell cultures

Calvaria from 3-d-old Swiss Webster mice were removed aseptically, rinsed in Dulbecco's minimum essential medium (DMEM), and incubated in a solution of 2 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN) in DMEM at 37°C for 20 min. The solution was discarded and the calvaria were reincubated in fresh collagenase-containing solution for 100 min. The cells were collected by centrifugation, resuspended in DMEM containing 10% fetal bovine serum (FBS, Hyclone, Logan UT) and plated in 75-cm^2 flasks at a density of 3 x 10^6 cells/flask. Twenty-four-h later the medium was replaced with DMEM containing 10% platelet-poor plasma (Cocalico, Reamstown, PA) to inhibit growth of fibroblasts (6).

The cells were subcultured 6-7-d later by trypsinization and then seeded at a density of 20,000 cells/well on 96-well plates in medium consisting of DMEM containing 10% FBS. After allowing the cells to attach overnight, the cultures were used for mitogenic assays.

Mitogenic assays

Osteoblasts plated in 96-well plates were incubated for 24 h with DMEM alone. One μl of each reconstituted column fraction was then added to each of 6 wells. BSA (2 μg/ml) from the column samples was too low to stimulate mitogenesis. After a 24-h incubation, 1 μC of [*methyl*-^{3}H]thymidine (5 Ci/mmol, Amersham, Arlington Hgts., IL) was added to each well. The incubation was terminated 4-h later by removal of the medium. Cells were treated with trypsin for 1 h and harvested onto filter paper with a cell harvester (Skatron, Sterling, VA). Thymidine incorporation was quantitated by liquid scintillation counting.

Mitogenic assays with human skin fibroblasts were performed in the same manner as described for the osteoblasts except that 5,000 cells were plated per well.

Cell counting

Cells were plated in 24-well plates with 120,000 cells/well. The same protocol was followed as for the thymidine incorporation assay except that the cultures were incubated incubated with samples for 48 h. Following trypsin treatment, the cells were counted with a hemocytometer.

Enzymatic digestion of column samples

Column samples were resuspended in 0.1 M HEPES, pH 8.4, containing 2 mM $CaCl_2$. Samples were split into 2 aliquots: one was incubated with 1 mg of nonspecific *S. griseus*

protease (Sigma, St. Louis, MO) covalently linked to agarose; the other was incubated with an equivalent amount of enzyme preparation that had been inactivated by boiling for 10 min. After an overnight incubation at 37°C, the supernatants were tested for mitogenic activity.

Results

Gel filtration of the ultrafiltrate revealed several peaks of mitogenic activity (Fig. 1). The activity eluting at or near the void volume of the column was probably due in part to known bone-cell mitogens such as the IGF's. The 5,000-dalton cutoff of the ultrafiltration membrane is only a nominal value. In addition, there were peaks of mitogenic activity with estimated molecular weights of 1,600, 1,050, 770, and 400-450. The 1,600-dalton peak of mitogenic activity was sometimes resolved into 2 species. These peaks were observed in 5 different chromatographic separations of distinct extracts of bone. Thymidine incorporation data were corroborated by direct cell counting (Data not shown). In particular, Fraction 39, which inhibited mitogenesis, did not kill the cells, but the number was reduced compared to Fraction 25, which had no effect on thymidine incorporation.

To determine whether the low-molecular-weight mitogens were peptides, they were treated with a nonspecific protease (Fig. 2). Mitogenic activity in the void volume (>7,000

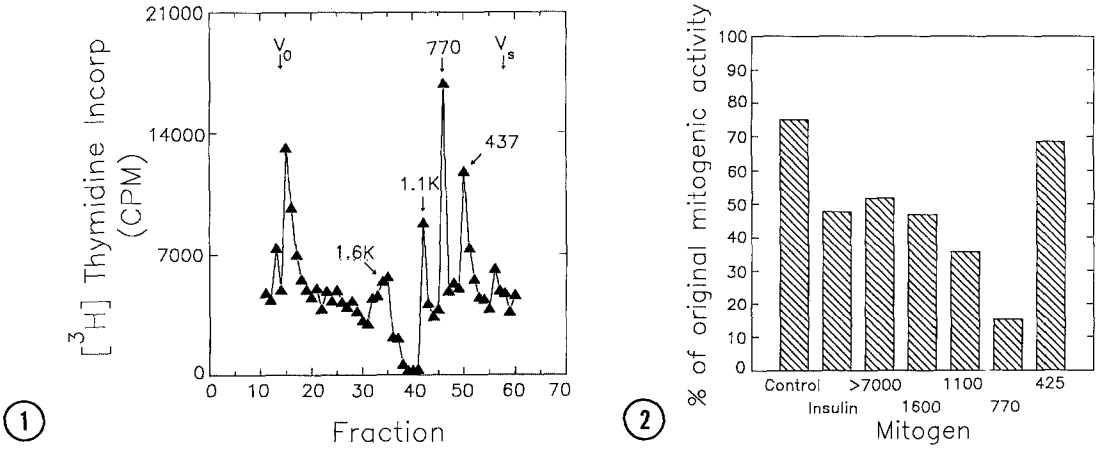

Figure 1. Gel filtration chromatography of an acid extract of neonatal mouse calvaria. One μl (out of 20 μl) of the resuspended residue was added to each of 6 wells of mouse osteoblast-like cells. The mean counts per minute for the 6 wells is plotted. The estimated molecular weights of the peak fractions is indicated.

Figure 2. Proteolytic digestion of low molecular-weight mitogens. The residue from selected column fractions was redissolved in HEPES buffer and equal aliquots were incubated with a nonspecific protease or heat-inactivated protease overnight. The samples were then assayed for their ability to stimulate osteoblast mitogenesis. Data are expressed as the percent decrease in mitogenic activity of samples treated with active enzyme to samples treated with inactive enzyme. *Control* refers to 2 column fractions that did not stimulate mitogenesis. The values on the *X-axis* refer to the molecular weights of the mitogens estimated from gel filtration chromatography.

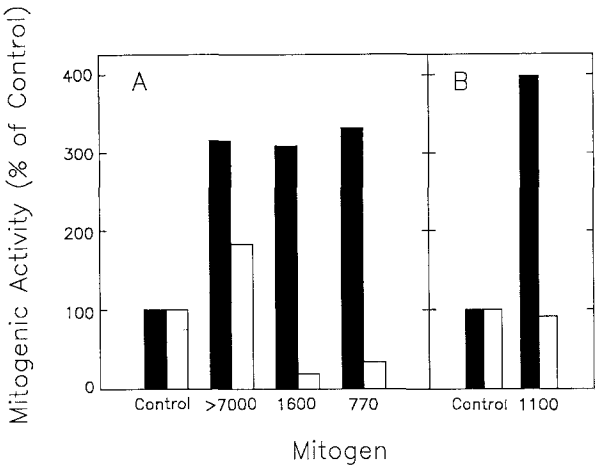

Figure 3. Effect of bone-derived mitogenic peptides on fibroblast and osteoblast proliferation. The data are plotted as the percent of the mitogenic activity of control fractions selected for their lack of effect on osteoblast mitogenesis. *A*, Two controls equivalent to Fractions 28 and 59 of Fig. 1 were used. *B*, One control equivalent to Fraction 39 was used. In the experiment depicted, insulin stimulated osteoblast mitogenesis 2.6-fold and fibroblast mitogenesis 3.3-fold. The values on the *X-axis* refer to the molecular weights of the mitogens estimated from gel filtration chromatography. Osteoblasts, *solid* bars; fibroblasts, *open* bars.

Da), column fractions that did not stimulate mitogenic activity, and insulin were treated identically. Thymidine incorporation for the control fractions decreased approximately 25% after enzyme treatment, whereas the activity of insulin was reduced by about 50%. As anticipated, the void volume mitogenic activity was diminished by enzyme treatment. Three of the 4 low-molecular-weight mitogens showed reduced ability to stimulate thymidine incorporation. The loss of mitogenic activity was variable, being greatest for the 770-dalton material.Only the 400-450-dalton material was unaffected by protease treatment.

Fibroblasts are a common contaminant of the osteoblastic cell cultures and their proliferation is regulated by many of the same growth factors as osteoblasts (Reviewed in 7). Therefore, we examined the response of fibroblasts to the low molecular-weight mitogenic peptides (Fig. 3). As anticipated, the void volume material (>7,000 Da) stimulated proliferation of both cell types. However, the low molecular weight peptides only enhanced osteoblast mitogenesis. Both the 1,600- and 770-dalton peptides appeared to inhibit fibroblast proliferation. The 1,100-dalton peptide had no effect on the fibroblasts.

Discussion

We have demonstrated the existence of low-molecular-weight peptides, extracted from rapidly mineralizing bone, that stimulate bone proliferation. The estimated molecular weights of these peptides, 1,600, 1,050, and 770, makes them far smaller than the smallest well-

characterized bone-cell mitogen, the 7,500-dalton IGF-I (1). Whether these peptides are unique or are previously discovered peptides with other functions is unknown. Sequence analysis of purified peptides will be required for complete identification.

In contrast to the well-characterized osteoblast growth factors (1,7), the three low-molecular-weight mitogenic peptides did not stimulate fibroblast proliferation. Indeed, fibroblast mitogenesis appeared to be inhibited under the experimental conditions employed. This selective action argues against the mitogenic peptides being fragments of known growth factors.

The hypothesis that bone contains low-molecular-weight growth factors determined the partial purification scheme. Purification of bone-bioactive peptides often employs dialysis or ultrafiltration as an early step. Low molecular weight material typically is discarded. However, we specifically saved this material and assessed mitogenic activity following gel filtration chromatography.

The cellular origin of these peptides is unknown. Certain cell types can be eliminated. This newly formed bone has little or no marrow space suggesting that cells of the immune system and hematopoietic precursors are not the source. Furthermore, fibroblasts are unlikely to secrete peptides of this small size since they only have a constitutive secretory pathway (8). Many bone-cell mitogens have been detected in bone matrix (1) and it is possible that any or all of these small peptides are plasma-derived, binding to the matrix.

Peptides of the size demonstrated by these experiments are commonly exported via the regulated secretory pathway of neuronal, endocrine or exocrine cells (9). Indeed, bone is innervated with peptidergic neurons that contain VIP, (MW 3,300) and CGRP (MW 3,800) (10,11). However, neither of these peptides could account for the data shown here. Extracts of a rat clonal osteoblast cell line have detectable peptidyl-glycine α-amidating monooxygenase activity (Bertelsen A.M., Drivdahl R., and Birnbaum, R.S., unpublished observations), a marker for the regulated secretory pathway (12). We speculate that the osteoblast may be one source of these mitogenic peptides and that this cell has a regulated as well as constitutive secretory pathway.

Acknowledgments

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