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A PROTEOGLYCAN FORM OF MACROPHAGE COLONY-STIMULATING FACTOR THAT BINDS TO BONE-DERIVED COLLAGENS AND CAN BE EXTRACTED FROM BONE MATRIX

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SUMMARY: We previously found that the human osteoblastic cell line MG-63 produces two molecular types of macrophage colony-stimulating factor (M-CSF). One is an 85-kD M-CSF, and the other is a proteoglycan form of M-CSF (PG-M-CSF) that has a binding affinity to type V collagen. The latter type of M-CSF showed dose-dependent binding to wells coated by pepsin-extracted bone collagens, whereas the 85-kD M-CSF did not. Immunoblot analysis of urea-extracted bone M-CSF revealed the presence of PG-M-CSF. PG-M-CSF contained in bone matrix may have physiological importance in the bone metabolism.

Several recent studies on a hereditary osteopetrotic mouse (op/op) revealed the importance of macrophage colony-stimulating factor (M-CSF) in bone metabolism. The op/op mouse has a single base pair insertion in the coding region of the M-CSF structural gene (1) that results in the inability to produce functional M-CSF (2). Injection of M-CSF reverses the osteoclast deficiency in the op/op mouse (3). These data indicated that M-CSF is required for osteoclast formation in vivo.

M-CSF was originally purified from human urine and shown to be a disulfide-linked homodimer of a 43-kD subunit with an apparent

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molecular weight of 85-kD (4). We previously reported that the human osteoblastic cell line MG-63 produced two molecular forms of M-CSF (5). One form is an 85-kD M-CSF, which is the major species of M-CSF in body fluids (blood and urine) (6). The other is a proteoglycan form of M-CSF (PG-M-CSF), which we recently identified in the conditioned medium of Chinese hamster ovary cells transfected with a 4.0-kb M-CSF cDNA (7). PG-M-CSF constitutes a considerable portion of the M-CSF produced by MG-63 cells and had almost the same colony-stimulating activity as 85-kD M-CSF (5), suggesting that it has an important role in bone metabolism.

PG-M-CSF is a heterodimer consisting of a 43-kD subunit and a 150-200 kD subunit that is a proteoglycan carrying a chondroitin sulfate glycosaminoglycan chain with a core protein of 100-kD (7). It can bind to type V collagen through its glycosaminoglycan chain with a retention of its biological activity (8). Type V collagen is usually detected in mesenchymal tissues such as bone, co-distributed with interstitial collagens (type I, II, and III collagen) (9). Rhodes et al. reported that the pepsin-extracted collagens of bone contained about 4 % type V collagen (10).

In this study, we describe the binding affinity of PG-M-CSF to pepsin-extracted bone collagens and the extraction of PG-M-CSF from human bone.

MATERIALS AND METHODS

Pepsin-extraction of collagens from human bone

Bone collagens were extracted according to the method described by Miller et al. with slight modifications (11). Eight grams of lyophilized bone was powdered and washed five times with 250 ml of 50 mM Tris/HCl (pH 7.4) containing protease inhibitors (5 mM ethylene diamine tetraacetic acid (EDTA), 5 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.36 mM pepstatin A) (solution A) to remove blood components. After washing, bone pieces were decalcified in 200 ml of 0.5 M EDTA, follwed by treatment with 200 ml of solution A containing 2 M urea to remove noncollagenous proteins such as proteoglycan. After discarding the supernatant, collagens were extracted by mixing the bone in 100 ml of 0.5 M acetic acid containing 80 mg of pepsin (Wako Pure Chemical Industry Co. Ltd.) for 24 hours.

Pepsin in the extract was inactivated by the addition of pepstatin A at a concentration of 5 μg/ml, and this solution was reduced to 5 ml by using an ultra-filtration membrane (Centricut 10, Kurashikiboseki Co., Ltd.) with a 10,000 molecular weight cut-off filter. These concentrated pepsin-extracted collagens were used for the modified ELISA described below.

All procedures were performed at 4 °C. Bone was obtained from the lumbar vertebra of a 55-year old deceased female patient after obtaining the consent of her family.

Preparation of PG-M-CSF from MG-63 conditioned medium

PG-M-CSF was purified from the conditioned medium of MG-63 cells as previously reported (**8**).

An enzyme-linked immunosorbent assay (ELISA) and its modification

The amount of M-CSF was determined by an ELISA as previously reported (**12**). The results were obtained as optical density (OD) at a wavelength of 492 nm.

A modified ELISA was established to investigate the binding between pepsin-extracted bone collagens and two types of M-CSF, in which 0.1 ml of the test subject was used for coating the plate instead of the first anti-M-CSF Ab. Before the coating, pepsin-extracted collagens of bone were neutralized with 1 M NaOH. The subsequent steps were the same as those for ELISA. The results are expressed as the means ± standard deviations of triplicate assays.

Extraction of noncollagenous proteins from bone by urea

Bone matrix proteins were extracted by the method reported by Termine et al. (**13**). After washing, 8 g of bone pieces were mixed in 100 ml of solution A containing 7 M urea (to degenerate collagens) at 4 °C for 48 hours. The supernatant was reserved, and the residue was reextracted under the same extraction conditions with the addition of 0.5 M EDTA. These extracts were combined and concentrated to 5 ml as described above. This concentrated bone-extract was used in the detection of PG-M-CSF described below.

DEAE-Sephacel column chromatography

One milliliter of the concentrated bone-extract was applied to 1 ml of DEAE-Sephacel (Pharmacia) which had previously been equilibrated with solution A containing 7 M urea (solution B). After washing with 10 ml of solution B, the column was eluted with 2 ml of solution B containing 0.1, 0.2, 0.3, 0.4, 0.5, or 0.6 M NaCl. Each eluate was dialyzed against PBS containing 1.5 % BSA, and then the M-CSF was quantified.

Chondroitinase AC treatment

The 0.4 - 0.6 M NaCl eluates from the DEAE-Sephacel were combined and digested with 10 mU/ml of chondroitinase AC (EC 4.2.2.5; Seikagaku Kogyo) in 0.1 M Tris/HCl (pH7.2) and 30 mM calcium acetate in the presence of protease inhibitors as previously reported (**5**).

Immunoblot analysis of M-CSF in the urea-extracted bone proteins

Eighteen milligrams of IgG prepared from the serum of a horse immunized with purified native human M-CSF was coupled 1 ml of formyl-cellulofine (Seikagaku Kogyo) and used as the antibody (Ab)-bound affinity column. Four milliliters of the concentrated bone-extract was dialyzed against solution B containing 0.1 M NaCl and eluted through 10 ml of DEAE-Sephacel with 20 ml of solution B containing 0.3 M NaCl. Secondly, the column was eluted with 20 ml of solution B containing 0.6M NaCl. Each eluate was dialyzed against solution A and applied to the Ab-bound affinity column. Elution was performed with 3.5 M potassium thiocyanate in PBS (pH 7.4), and the eluate was immunoblotted under reducing condition according to the previously described method (**6**).

Statistical analyses
Statistical comparisons of independent sample means were made by Student's t test.

RESULTS AND DISCUSSION

Each well of the microtiter plate was coated with pepsin-extracted bone collagens, and the binding of both 85-kD rhM-CSF and purified PG-M-CSF to the well was studied (Figure 1A). PG-M-CSF bound to pepsin-extracted collagens in a dose-dependent manner, whereas 85-kD rhM-CSF had no binding activity. To determine if PG-M-CSF bound to type V collagen in the pepsin-extracted collagens, the well was incubated with PG-M-CSF in the presence of increasing amounts of soluble type V collagen (Figure 1B). Soluble type V collagen markedly inhibited the binding of PG-M-CSF to surface-adsorbed pepsin-extracted collagens, whereas type IV collagen (control) weakly inhibited it. These data suggested that PG-M-CSF bound to native human bone collagens, especially to type V collagen in the bone matrix.

Since PG-M-CSF has a higher affinity for an ion exchange matrix as compared with 85-kD M-CSF , it is easily separated from 85-kD M-CSF by DEAE-Sephacel (7). 85-kD M-CSF was completely eluted from the column at 0.4 M NaCl, whereas PG-M-CSF bound tightly and was eluted at a higher NaCl concentration (0.6 M NaCl). We extracted noncollagenous proteins from human bone by using 7 M urea and eluted the bone-extract through a DEAE-Sephacel column with solution B containing 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M NaCl. The M-CSF level in each fraction was measured by ELISA, and the amount of M-CSF was indicated as OD_{492} (Figure 2A). The fractions eluted by 0.4, 0.5, and 0.6 M NaCl were all positive for M-CSF, suggesting that the bone-extract contained PG-M-CSF. To further examine the M-CSF in the fractions eluted by 0.4, 0.5, and 0.6 M NaCl, we digested them with chondroitinase AC, which degrades chondroitin sulfate, and eluted the product through a DEAE-Sephacel column again (Figure 2B). Chondroitinase AC

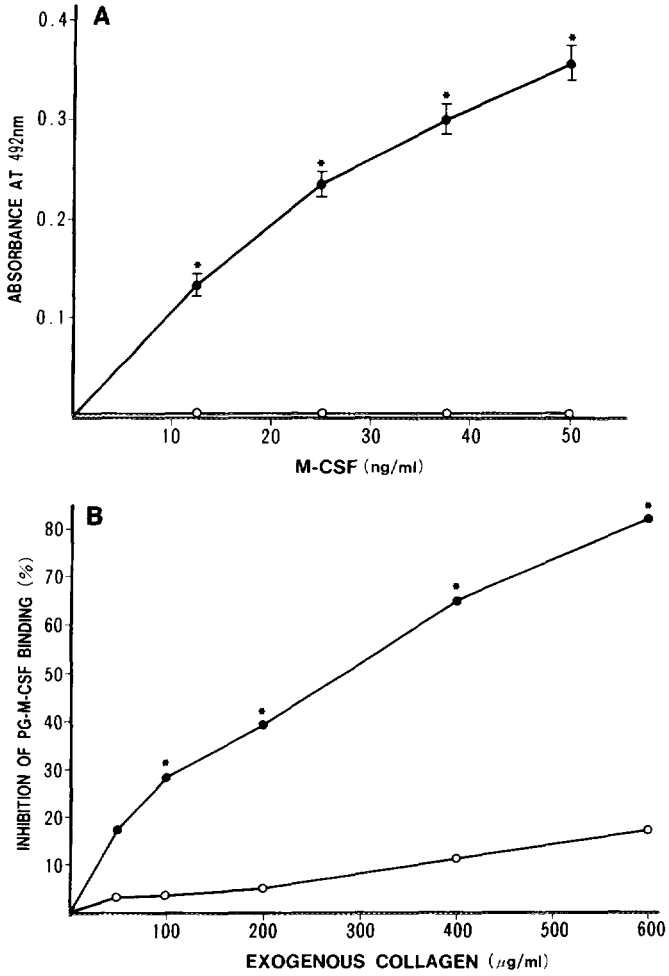

Figure 1. Binding of M-CSFs to pepsin-extracted bone collagens
A. Each well of the microtiter plate was coated with 0.1 ml of the concentrated pepsin-extracted bone collagens. The modified ELISA was performed as described in MATERIALS AND METHODS to quantitate the amount of M-CSF binding to the well. Data are reported as OD492. Samples showing a significant differece from the controls at $p<0.05$ are indicated (*). Closed circles, PG-M-CSF; open circles, 85-kD rhM-CSF.
B. Wells coated with the pepsin-extracted bone collagens were incubated with 50 ng/ml PG-M-CSF in the presence of increasing amounts of purified type V or type IV collagen (Wako Pure Chemical Industry Co., Ltd.). The inhibition percentage of the binding is indicated, taking the OD492 of the well incubated with 50 ng/ml PG-M-CSF in the absence of exogenous type V collagen as 100 %. Closed circles, type V collagen; open circles, type IV collagen.

treatment resulted in the loss of binding activity of the M-CSF to DEAE-Sephacel in the presence of 0.4 - 0.6 M NaCl. This result suggested that PG-M-CSF in the fractions was degraded by chondroitinase AC and lost its affinity to DEAE-Sephacel at the

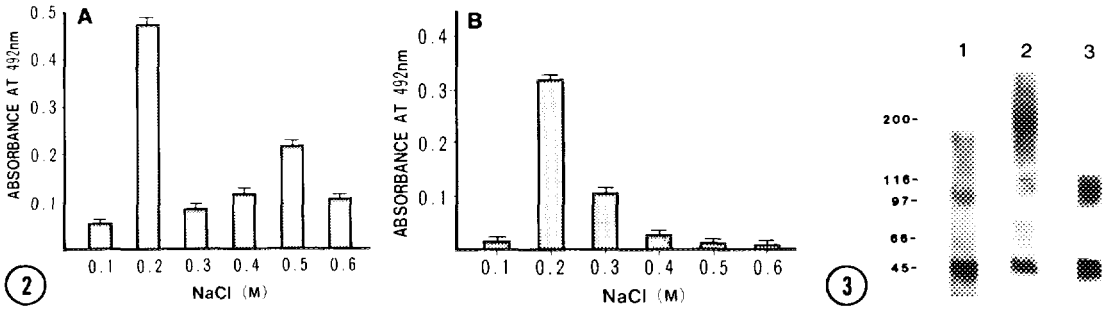

Figure 2. DEAE-Sephacel column chromatography of urea-extracted bone M-CSF and ELISA of the fractions
A. One milliliter of the urea-extracted bone M-CSF was applied to 1 ml of DEAE-Sephacel and eluted with 2 ml of 50 mM Tris/HCl (pH 7.4) containing 0.1, 0.2, 0.3, 0.4, 0.5, or 0.6 M NaCl. Each fraction was dialyzed against PBS with 1.5 % BSA, and then the amount of M-CSF in each fraction was determined by ELISA. M-CSF levels are shown as OD492.
B. One milliliter of each fraction eluted by 0.4, 0.5, or 0.6 M NaCl in Figure 2A was combined and treated with chondroitinase AC. DEAE-Sephacel column chromatography and ELISA were performed as described above.

Figure 3. Immunoblot analysis of the urea-extracted bone M-CSF
Four milliliters of the bone extract was sequentially applied to a DEAE-Sephacel column and an Ab-bound affinity column as described in MATERIALS AND METHODS. The eluate was analyzed by Western blotting under the reducing condition using anti-M-CSF Ab. Molecular weight standards (in kD) are shown on the left. Lane 1, M-CSF in 0.1 - 0.3 M NaCl eluate; lane 2, M-CSF in 0.4 - 0.6 M NaCl eluate; lane 3, M-CSF in 0.4 - 0.6 M NaCl eluate after chondroitinase AC treatment.

high concentration of NaCl, because the affinity of PG-M-CSF is conferred through its chondroitin sulfate glycosaminoglycan chain (8). M-CSF eluted by 0.1 and 0.2 M NaCl in Figure 2A may be a contaminating 85-kD M-CSF carried from the blood, or a degradation product of PG-M-CSF which has lost affinity for DEAE-Sephacel during the extraction process. The data indicating that PG-M-CSF is proteolytically cleaved to form a 85-kD product that is identical in size to 85-kD M-CSF (7) supports the latter speculation. PG-M-CSF in the bone matrix may be converted to 85-kD M-CSF by proteases derived from the cellular components of bone during the extraction process.

Immunoblot analysis under the reducing condition of the M-CSF in the 0.4 - 0.6 M NaCl eluate showed that it consisted of 43-kD subunit and high molecular weight subunit (Figure 3). The latter

subunit was sensitive to chondroitinase AC, indicating that it was definitely PG-M-CSF (7). Some growth factors such as fibroblast growth factor (14) and granulocyte-macrophage colony-stimulating factor (15) are reported to interact with the extracellular matrix (ECM). Insulin binds to type V collagen with retention of its activity (16). These findings indicate that ECM serves as a reservoir for growth factors and controls their concentration in the microenvironment. A recent study has revealed that the bone matrix is a rich source of growth factors (17) such as transforming growth factor-β, bone-derived growth factor-II (β_2-microglobulin), platelet-derived growth factor, and insulin-like growth factor (IGF)-I. In the same manner, PG-M-CSF may be stored in the bone matrix, where it binds to type V collagen.

Osteoclasts are believed to be derived from common progenitor cells of osteoclasts and macrophages in the presence of 1α, 25-$(OH)_2$ vitamin D_3 and M-CSF (18). It was reported that op/op mouse osteoblasts supported the differentiation of osteoclasts in the presence of exogenous 85-kD M-CSF, although a concentration of 85-kD M-CSF much higher than its normal serum level was required for osteoclast formation in vitro (19). The binding between PG-M-CSF produced by osteoblasts and type V collagen in the bone matrix may increase the local concentration of M-CSF, thus allowing the osteoclasts to differentiate.

The role of PG-M-CSF in bone metabolism in vivo still remains to be clarified.

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