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CLINICAL INVESTIGATION

Identification of a low molecular weight inhibitor of osteoblast mitogenesis in uremic plasma

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Identification of a low molecular weight inhibitor of osteoblast mitogenesis in uremic plasma. A low molecular weight inhibitor of cartilage sulfation has been detected in the plasma of dialysis patients. Preliminary studies of this inhibitor have suggested that it may have a role in decreasing bone mass, possibly by suppressing bone cell proliferation. Since the in vitro bioassay of crude sulfation inhibitor preparations is relatively nonspecific, we investigated whether there might also be an inhibitor of osteoblast mitogenesis in uremic plasma. We fractionated plasma and plasma ultrafiltrates from dialysis patients by gel filtration chromatography and looked for inhibition of mitogenesis in cultured osteoblasts. Material from fractions with a molecular weight range of 750 to 900 inhibited osteoblast mitogenesis. The inhibitory effect, however, could be overcome with serum or insulin-like growth factor-I, suggesting that the mechanism of inhibition was not growth factor dependent. Further characterization of the inhibitor revealed that it was not a peptide or a polar lipid. We conclude that uremic plasma contains a bone cell mitogenic inhibitor which may have a role in regulating bone remodeling in adults and bone growth in children.

Although growth factors have received considerable attention in the regulation of cell proliferation, growth inhibitors may play an equally important role [1–6]. One such inhibitor, with an estimated molecular weight of 940, is elevated in the plasma of uremic patients [6]. This low molecular weight inhibitor was identified by its ability to blunt insulin-like growth factor-I (IGF-I) stimulated cartilage sulfation, an effect which may be secondary to reduced DNA and RNA synthesis [6]. It has been suggested that delayed bone growth in uremic children could be due, in part, to the suppressive effects of this inhibitor on growth plate cartilage [6], since this process is controlled primarily by localized production of IGF-I [7].

Recently, we demonstrated that elevated immunoreactive plasma IGF-I levels in uremic adults were closely correlated with increased bone formation [8], a finding consistent with the anabolic effects of IGF-I in cultured osteoblasts [9, 10]. We also found, in preliminary studies, that plasma levels of the 940-dalton uremic inhibitor were significantly elevated in dialysis patients with reduced bone mass, suggesting that the inhibitor may have suppressed bone mineralization [11]. Because osteoblast numbers on bone surfaces were greatly reduced in the patients with elevated inhibitor levels, we reasoned that the inhibitor may have decreased bone mass by inhibiting normal osteoblast proliferation. The purpose of the present study was to determine whether uremic plasma contains a low molecular weight inhibitor of osteoblast proliferation. The results indicate that a circulating inhibitor of osteoblast mitogenesis is present in uremic plasma and that it acts primarily to suppress basal cell growth by a mechanism that is independent of IGF-I receptor stimulation.

Methods

Plasma preparations

For the initial studies a pool was prepared from the plasma of 34 chronic hemodialysis patients. Subsequently, plasma ultrafiltrates were collected from five uremic patients on hemodialysis. The ultrafiltrates were obtained by collecting 500 ml of fluid from each patient while recirculating 500 ml of dialysate on the venous side of a cuprophane membrane dialyzer (molecular wt cutoff: 5,000). Each one liter volume was taken to dryness with a Speed-Vac concentrator (Savant) and reconstituted in 10 ml of 0.1 M ammonium formate, pH 7.0.

Gel filtration chromatography

A 10 ml aliquot of pooled plasma was fractionated at 4°C using Sephadex G-25 (superfine) equilibrated in 0.1 M ammonium formate, pH 7.0. Ten milliliter fractions were collected, taken to dryness and reconstituted in distilled water for assay. The column was calibrated with the following molecular weight standards: blue dextran (V_o), [^{125}I]procalcitonin [1-12] (1650 daltons), [^{125}I]procalcitonin [52-57] (945 daltons), and [^{125}I]thyrotropin-releasing hormone (340 daltons). The concentrated plasma ultrafiltrates were chromatographed in the same manner. Active fractions from chromatography of the ultrafiltrate preparation were pooled and further characterized.

Osteoblast mitogenic assays

Inhibition of mitogenesis was determined using either primary cultures of osteoblast-like cells (osteoblasts and osteoblast progenitors) from neonatal rat calvaria or U-2 human osteosarcoma cells (U-2 OS; ATCC, Rockville, Maryland, USA). Normal osteoblast-like cells were released from calvaria of 2- or 3-day-old rat pups by collagenase digestion, plated onto

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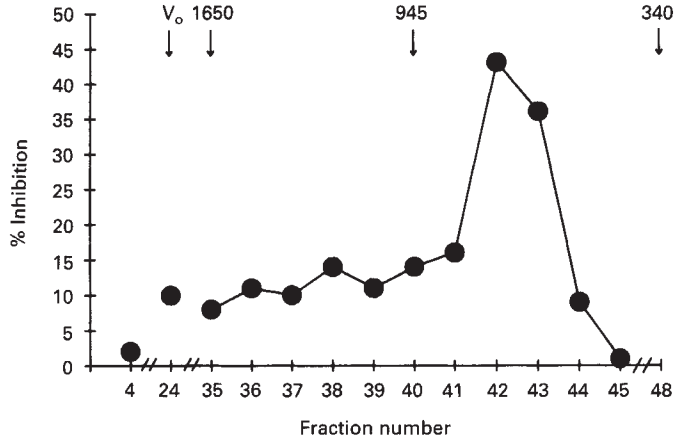

Fig. 1. *Gel filtration chromatography of pooled uremic plasma.* Ten ml of plasma were fractionated on Sephadex G-25 and fractions assayed for inhibition of osteoblast mitogenesis using cultures of rat calvaria. Results are expressed as a percentage of inhibition of cells stimulated by 5% FBS. Each point represents the mean of 5 separate mitogenic assays.

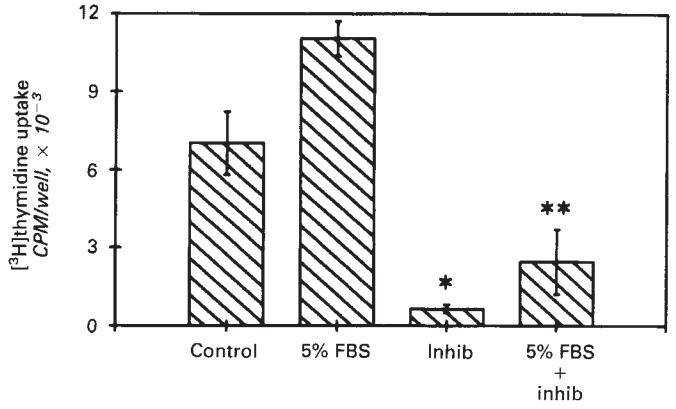

Fig. 2. *Inhibition of mitogenesis in U-2 OS cells.* The amount of inhibitor (INHIB) tested was 3% of the total volume of culture medium (100 μl). Values are the mean ± SD of 3 experiments. *$P < 0.05$ vs. control; **$P < 0.05$ vs. FBS-stimulated.

96-well plates, and grown in BGJ_b medium (GIBCO) containing 10% fetal bovine serum (FBS) for 24 hours in a 5% CO_2-air atmosphere and then incubated in serum-free BGJ_b medium for an additional six to eight hours. The U-2 OS cells were treated in a similar manner following trypsinization of stock cultures except that they were grown in McCoys 5a medium (GIBCO) containing 5% FBS before incubation with serum-free medium.

Following incubation with serum-free medium, mitogenesis was stimulated by transferring cultures into medium containing 5% FBS or human recombinant IGF-I (50 ng/ml, Bachem) and samples to be tested for inhibitory activity were added at this time. After overnight incubation, [methyl-^{3}H]thymidine (1 μCi/well; Amersham) was added for a four-hour labelling period. The cells were then washed, collected onto paper discs by a cell harvester (Skatron) and the radioactivity quantified in a liquid scintillation counter.

Proteolytic digestion of inhibitor preparations

Aliquots of inhibitory material were incubated with 1 Unit of insoluble protease (from *Strep.* griseus attached to carboxymethyl cellulose; Sigma) for 4 and 24 hours in HEPES buffer containing 2 mM calcium at 37°C. The supernatants from the digestions were assayed for their effect of U-2 OS cell mitogenesis. As a control, inhibitory material was incubated with the enzyme preparation after the protease had been inactivated by boiling.

Chloroform extraction

Equal volumes of chloroform and mitogenic inhibitory material were combined and shaken by hand for two minutes. After allowing the mixture to stand for 30 minutes at room temperature, it was centrifuged at 4°C. The aqueous layer was removed and following reextraction with chloroform, concentrated by Speed-Vac. The chloroform extracts were pooled and dried under nitrogen. Residues from the aqueous and chloroform fractions were reconstituted for the mitogenic assay. Water controls were extracted and tested in an identical manner.

Results

Chromatography of pooled uremic plasma revealed mitogenic inhibitory activity in normal osteoblasts in a molecular weight range of 750 to 900 daltons (Fig. 1). This compares well with the 940 dalton (range of 800 to 1100 daltons) inhibitor of cartilage sulfation previously identified in uremic plasma [6], using the same chromatographic procedure. Gel filtration was necessary to separate this small inhibitor from growth factors that stimulate osteoblast proliferation, such as IGF-I [10], and other growth inhibitors [6, 12]. The inhibitory material was not cytotoxic since > 90% of the cells excluded trypan blue. Inhibitory material of the same size was also recovered after gel filtration of plasma ultrafiltrates (data not shown) and was used in the subsequent characterizations.

The low molecular weight inhibitor decreased DNA synthesis in the human U-2 OS osteoblast-like cells (Fig. 2), in both stimulated and unstimulated cells. The observation that thymidine uptake in FBS-stimulated cells was not suppressible to the same level as the unstimulated cells suggested that inhibition of cell growth may not be growth factor specific. To evaluate this possibility, mitogenic inhibition of normal osteoblasts stimulated by IGF-I (50 ng/ml) was compared to inhibition of unstimulated cells (Fig. 3). Inhibition of IGF-I-stimulated mitogenesis (70%) was similar to inhibition in the unstimulated controls (72%). That is, IGF-I stimulated osteoblast mitogenesis by about 1.8-fold, regardless of whether the inhibitory material was present or not.

Because previous studies demonstrated that some low molecular weight uremic inhibitors of DNA synthesis are peptides [5], we evaluated this possibility by testing our inhibitory material following protease digestion. As shown in Figure 4, prolonged incubation with a nonspecific protease did not alter the inhibitory activity in the mitogenic assay. Since the inhibitor was not a peptide, we attempted to extract the material with chloroform to assess the possibility that the active substance might be a polar lipid (Fig. 5). Uremic plasma has been shown to contain polar lipids that inhibit erythropoiesis [13]. In the mitogenic assay no inhibitory activity was extracted into chloroform and all of the inhibition was recovered in the aqueous layer.

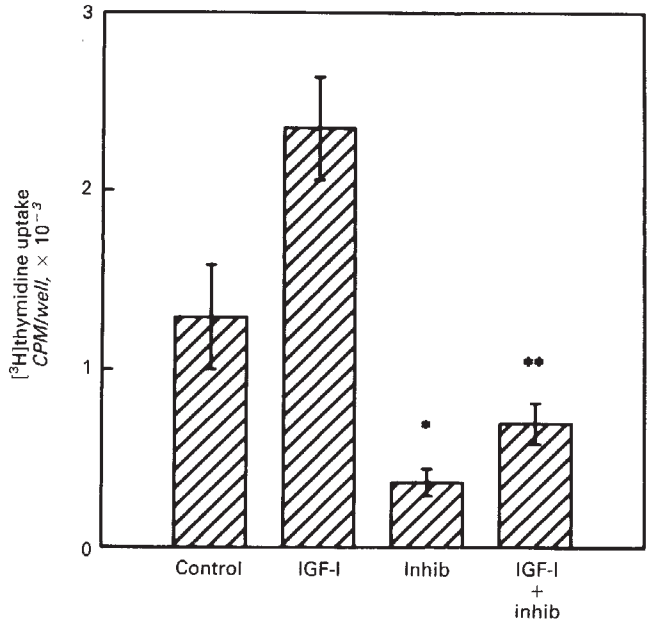

Fig. 3. *Inhibition of mitogenesis in IGF-stimulated rat osteoblasts.* IGF-I, 50 ng/ml, was used to stimulated mitogenesis. The amount of inhibitor (Inhib) tested was 3% of the total volume of culture medium (100 μl). Values are the mean ± SE of 6 wells. $*P < 0.05$ vs. control; $**P < 0.05$ vs. IGF-stimulated.

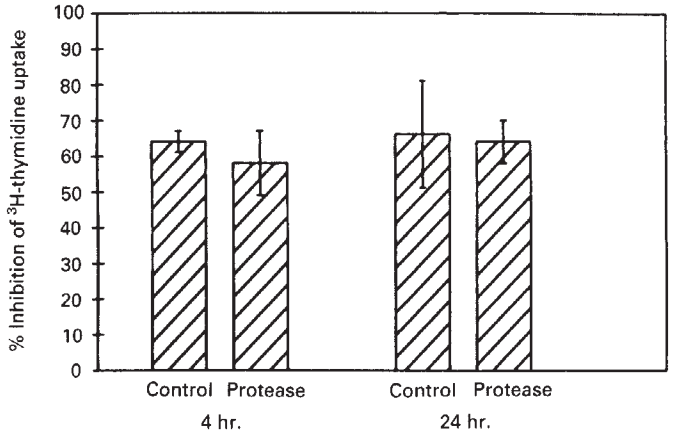

Fig. 4. *Lack of effect of protease digestion on inhibitory activity in U-2 OS cells.* The inhibitor preparation was treated with protease for 4 or 24 hours and then tested at a concentration of 3% (vol/vol). Values are the mean ± SD of 2 or 3 experiments.

Discussion

We have shown that a low molecular weight inhibitor of osteoblast mitogenesis exists in the circulation of uremic patients undergoing hemodialysis. The mitogenic inhibitor, which is not a peptide, is similar in size to the 940-dalton uremic inhibitor of cartilage sulfation and DNA synthesis identified by Phillips et al [6]. Although their inhibitor was originally reported to be partially inactivated by protease, a more recent characterization of this material by these authors suggests that it is not a peptide [14]. While the similar characteristics of these two inhibitors of DNA synthesis suggest that they may be the same

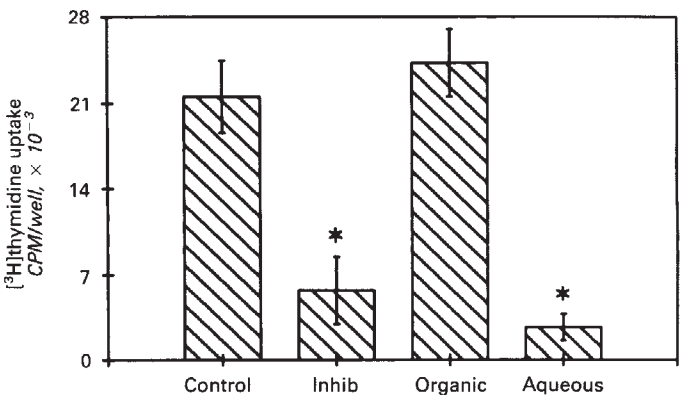

Fig. 5. *Effect of chloroform extraction on inhibitor activity in U-2 OS cells.* Inhib., untreated inhibitory material; Organic, chloroform phase; and Aqueous, aqueous phase. Control cultures contained 5% FBS and the inhibitor concentration tested was 3% (vol/vol) for all conditions. Values are for one experiment with the mean ± SD of 8 wells. $*P < 0.05$ vs. control.

molecule, the final comparison must await complete purification of both inhibitors.

Although the mechanism of inhibition was not fully examined, our finding that the inhibitor decreased basal (unstimulated) proliferation suggested that it may interfere with the autocrine regulation of cell growth. Since several osteoblast-derived growth factors, including IGF-I, stimulate osteoblast mitogenesis [15–17], inhibition of either their production or their receptor binding could decrease basal growth rates. When we tested serum-stimulated as well as IGF-I-stimulated cells, we found that cells treated with the inhibitory material were stimulated by the same amount as cells not exposed to the inhibitor. Thus, the inhibitor may decrease DNA synthesis by an effect which is not dependent on growth factor-receptor stimulation. Whether it decreases basal growth rates by decreasing production of osteoblast-derived growth factors remains to be determined.

Our recent preliminary study in uremic patients showing that elevated plasma levels of the 940 dalton inhibitor were associated with histological reductions in osteoblast number [11], together with the current in vitro data, suggest that inhibition of osteoblast proliferation may be an important cause of reduced bone mass in certain uremic patients with low turnover osteodystrophy [18]. The observation that IGF-I mitigates the mitogenic inhibitory effect in vitro may explain why some uremic patients with presumed elevations of the plasma inhibitor have normal or enhanced bone cell function when circulating levels of IGF-I and/or PTH, a stimulator of osteoblast-derived IGF-I [19], are also elevated [8]. In addition to its possible effects on osteoblast function in adults, the inhibitor may also have a role in causing delayed bone growth and short stature in uremic children. This could occur as a consequence of decreased osteoblast growth following the formation and proliferation of growth plate cartilage.

While the identity of the mitogenic inhibitor is unknown, it does not appear to be a protein or a protein fragment of known growth inhibitors [1–5]. In addition, failure to extract the inhibitory material in chloroform suggests that it is not the lipid-like inhibitor of erythropoiesis identified in uremic serum [13]. Whether the mitogenic inhibitor is related to the circulat-

ing low molecular weight acidic polyol inhibitor of nerve cell function [20] or to pentosidine [21], has not been ruled out.

In conclusion, we have isolated a circulating low molecular weight inhibitor of osteoblast mitogenesis in uremic patients. The inhibitor, which is not a peptide, acts to decrease basal cell proliferation but not DNA synthesis stimulated by IGF-I or serum. We suggest that this uremic inhibitor may have a permissive role in suppressing bone remodeling in adults and bone growth in children.

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References

1. Mashima K, Kimura T, Huang W, Yano K, Ashida Y, Yamagata Y, Miyazaki K, Yamashita J, Horio T: Multiple forms of growth inhibitors secreted from cultured rat liver cells: Purification and characterization. *J Biochem* 103:1020–1026, 1988
2. Blat C, Böhlen P, Villaudy J, Chatelain G, Golde A, Harel L: Isolation and amino-terminal sequence of a novel cellular growth inhibitor (inhibitory diffusible factor 45) secreted by 3T3 fibroblasts. *J Biol Chem* 264:6021–6024, 1989
3. Ervin PR Jr, Kaminski MS, Cody RL, Wicha MS: Production of mammastatin, a tissue-specific growth inhibitor, by normal human mammary cells. *Science* 244:1585–1587, 1989
4. LeBoeuf RD, Burn JN, Bost KL, Blalock JE: Isolation, purification and partial characterization of suppressin, a novel inhibitor of cell proliferation. *J Biol Chem* 265:158–165, 1990
5. Gutman RA, Huang AT: Inhibitor of marrow thymidine incorporation from sera of patients with uremia. *Kidney Int* 18:715–724, 1980
6. Phillips LS, Fusco AC, Unterman TG, del Greco F: Somatomedin inhibitor in uremia: *J Clin Endocrinol Metab* 59:764–772, 1984
7. Nilsson A, Isgaard J, Lindahl A, Dahlstrom A, Skottner A, Isaksson OGP: Regulation by growth hormone of number of chondrocytes containing IGF-I in rat growth plate. *Science* 233: 571–574, 1986
8. Andress DL, Pandian MR, Endres DB, Kopp JB: Plasma insulin-like growth factors and bone formation in uremic hyperparathyroidism. *Kidney Int* 36:471–477, 1989
9. Howard GA, Spencer EM: Somatomedins A and C directly stimulate bone cell proliferation in vitro, in *Endocrine Control of Bone and Calcium Metabolism* (vol 8A), edited by Cohn DV, Fujita T, Potts JT Jr, Talmage RV, Amsterdam, Excerpta Medica, 1984, pp. 86–89
10. Hock JM, Centrella M, Canalis E: Insulin-like growth factor I has independent effects on bone matrix formation and cell replication. *Endocrinology* 122:254–260, 1988
11. Andress DL, Phillips LS, Sherrard DJ: Elevated plasma somatomedin inhibitor in osteopenic diabetic dialysis patients. (abstract) *Kidney Int* 31:341, 1987
12. Phillips LS, Belosky DC, Young HS, Reichard LA: Nutrition and somatomedin. VI. Somatomedin activity and somatomedin inhibitory activity in serum from normal and diabetic rats. *Endocrinology* 104:1519–1524, 1979
13. Wallner SF, Vautrin RM: The anemia of chronic renal failure: Studies of the effects of organic solvent extraction of serum. *J Lab Clin Med* 92:363–369, 1978
14. Phillips LS, Goldstein S, Klein JD, Sulinski CM, El-Kebbi IM, Ziemer DC, Pollet RJ, Stults J: Uremic serum growth inhibitor. (abstract) *Clin Res* 38:33A, 1990
15. Heldin C-H, Johnsson A, Wennergren S, Wernstedt C, Betcholtz C, Westermark B: A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. *Nature* 319:511–514, 1986
16. Robey P, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB: Osteoblasts synthesize and respond to transforming growth factory-type beta (TGFβ) in vitro. *J Cell Biol* 105:457–462, 1987
17. Canalis E, McCarthy T, Centrella M: Isolation and characterization of insulin-like growth factor I (somatomedin C) from cultures of fetal rat calvariae. *Endocrinology* 122:22–27, 1988
18. Andress DL, Hercz G, Kopp JB, Endres DB, Norris KC, Coburn JW, Sherrard DJ: Bone histomorphometry of renal osteodystrophy in diabetic patients. *J Bone Miner Res* 2:525–531, 1987
19. McCarthy TL, Centrella M, Canalis E: Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures form fetal rat bone. *Endocrinology* 124:1247–1253, 1989
20. Man NK, Cueille G, Zingraff J, Drueke T, Jungers P, Sausse A, Boudet J, Funck-Brentano JL: Evaluation of plasma neurotoxin concentration in uremic polyneuropathic patients. *Proc Eur Dial Transpl Assoc* 5:164–170, 1978
21. Sell DR, Monnier VM: End-stage renal disease and diabetes catalyze the formation of a pentose-derived crosslink from aging human collagen. *J Clin Invest* 85:380–384, 1990