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INHIBITORY EFFECTS OF β_2-MICROGLOBULIN ON IN VITRO CALCIFICATION OF OSTEOBLASTIC CELLS

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Elevated levels of aluminum and β_2-microglobulin have been demonstrated in chronic dialysis patients. The role of aluminum in the pathogenesis of renal osteodystrophy has also been shown. We report on the effects of β_2-microglobulin on calcification in vitro using osteoblastic cells, clone MC3T3-E1. At concentrations comparable to those in plasma of chronic dialysis patients, both β_2-microglobulin and aluminum suppressed calcification while collagen synthesis and alkaline phosphatase activity were maintained. These observations may be related to the impaired bone mineralization frequently observed in chronic dialysis patients.
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Osteomalacia, one of the subtypes of renal osteodystrophy frequently observed in chronic dialysis patients, has been shown to be related to aluminum deposition originating from either dialysate and/or aluminum-containing phosphate binders (1).

On the other hand, following the identification of a major component of amyloid fibrils as β_2-microglobulin in a chronic dialysis patient with carpal tunnel syndrome (2), deposition of β_2-microglobulin in bone (3) and formation of urinary concretion consisting of β_2-microglobulin (4) in dialysis patients have been reported. In light of these observations, attention has been called to β_2-microglobulin deposition in various tissues in dialysis patients and its possible pathogenetic role in renal osteodystrophy.

We therefore tried to elucidate the effects of β_2-microglobulin as well as aluminum on bone metabolism in an in

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vitro setting by using a mouse calvaria-derived osteoblastic cells, clone MC3T3-E1 (5,6).

MATERIALS AND METHODS

Clone MC3T3-E1 cells, generously provided by Dr. Kodama (5,6), were cultured at 37°C in a humidified atomosphere of 5% CO_2 and 95% air by using Iscove's modified Dulbecco's medium (IMDM, Gibco, USA). To this medium, 10% of fetal calf serum (Flow Laboratories, USA), disodium β-glycerophosphate (final concentration :, 10mM) and ascorbic acid (50μg/ml) were added. At concentrations in the medium comparable to those in plasma of normal subjects and chronic dialysis patients, the effect of β_2-microglobulin on three distinct phases of bone metabolism, proliferation, differentiation and calcification of MC3T3-E1, was individually observed. To confirm the validity of this bioassay model, aluminum, the pathogenicity of which was clinically demonstrated, was evaluated as a control.

Aluminum chloride, which was added to the culture medium, was purchased from Wako Pure Chem., Japan. β_2-Microglobulin, on the other hand, was purified from uremic plasma as follows : ultrafiltrate was obtained from dialysis patients by using a special poly (methyl methacrylate)(PMMA) membrane ("Filtryzer"BK, Toray, Japan) with a molecular weight cut-off of 50,000-60,000 daltons, allowing the passage of β_2-microglobulin (7). This was fractionated through gel filtration (Sephadex G-50, Pharmacia, Sweden) followed by ion exchange chromatography (DEAE Sephacel, Pharmacia, Sweden), and the β_2-microglobulin fraction was dialyzed against 50mM CH_3COONH_4 and lyophilized. β_2-Microglobulin was identified by amino acid analysis and its purity was confirmed to be more than 96% by SDS-polyacrylamide gel electrophoresis. Concentrations of aluminum and β_2-microglobulin were measured by the atomic absorption analysis and enzyme immunoassay, respectively.

To evaluate the effects of aluminum and β_2-microglobulin on bone metabolism, 8×10^4 cells were plated in 35mm plastic dishes (Corning, USA) in 2ml of medium containing aluminum and β_2-microglobulin and the medium was refed every day. The confluent level of the cells was reached on day 3 after the start of cultivation. On day 5 to 7, collagen synthesis was qualitatively monitored by using an optical microscope after Masson's trichrome staining. Alkaline phosphatase (ALP) activity, which was used as an indicator of cell differentiation as well as collagen synthesis, appeared around day 3 to 5 and then linearly increased until around day 10, but leveled off afterwards. ALP activity per total protein was measured on day 14 by Kind-King's method (8) after rinsing cells with HEPES-buffered saline followed by sonication using an oscillator with ice-water cooling. Total protein was determined by the method of Sedmak and Grossberg (9). After a 14 day culture, the degree of calcification was evaluated semi-quantitatively after staining by the von Kossa's method (10). Prior to von Kossa's staining, the formation of hydroxyapatite was confirmed by an electron probe microanalyzer (Hitachi X-650, Japan) as shown in Figure 1, where the ratio of X-ray relative intensity of inorganic phosphorus (P) to calcium (CA) was demonstrated to be 0.6 to 1, similar to that in standard hydroxyapatite (Sigma, USA). To have a clue to the mechanism of the inhibitory effect of β_2-microglobulin on bone metabolism, the cells cultured in the presence or absence of β_2-microglobulin were treated with collagenase and sonicated. The concentration of

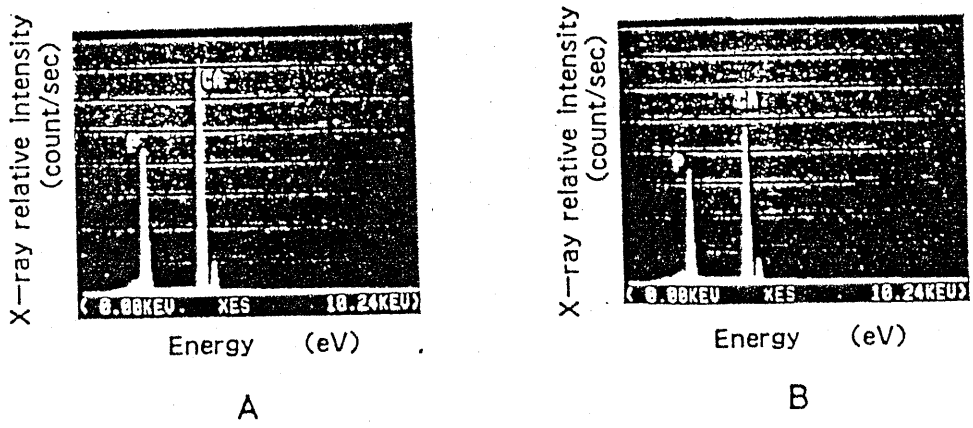

Figure 1. X-ray energy spectra of (A) hydroxyapatite (standard) and (B) highly mineralized area in tissue culture.

β_2-microglobulin in the supernatant fluid thus obtained was measured to determine the recovery rate of β_2-microglobulin from the cell layers.

RESULTS

Calcification. Under the conditions established, calcification was observed on day 10 after plating the cells. Determination of the degree of calcification could be performed on day 14. Figure 2 depicts four degrees of calcification on day 14. Mean contents of calcium measured after 20 day culture ranged from 0, 0.1, 0.3 and 0.5 mg/mg protein corresponding to qualitative assessment of

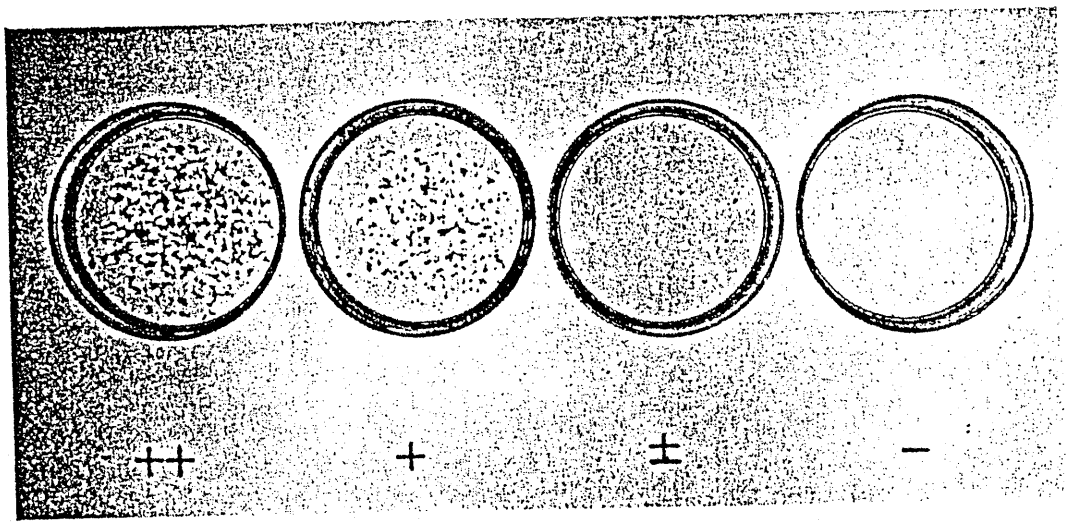

Figure 2. Photographs of plastic dishes, where different degree of calcification is visualized by von Kossa staining.

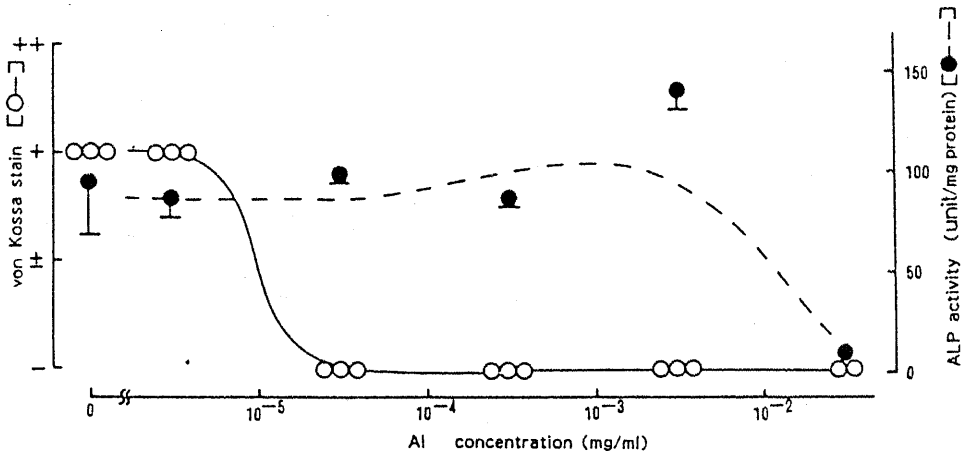

Figure 3. Effects of aluminum in the medium on ALP activity and calcification in a cell culture assay.

- , ± , + , ++ , respectively. The bioassay on calcification hereinafter was carried out using this qualitative classification shown in Figure 2.

Effects of aluminum. In Figure 3, the effects of aluminum on ALP activity and calcification as a function of aluminum concentration in the medium are depicted. At a concentration of $3x10^{-6}$mg/ml, which corresponds to plasma aluminum level of normal subjects (less than 10^{-5} mg/ml), neither ALP activity nor calcification were suppressed. However, as aluminum concentration was increased to the range which corresponds to plasma aluminum levels in chronic dialysis patients ($2\text{-}20x10^{-5}$mg/ml) (11), calcification was thoroughly suppressed without any change in ALP activity. At an aluminum concentration of the order of 10^{-2}mg/ml, cell proliferation itself as well as ALP activity was prevented.

Effects of β_2-microglobulin. The inhibitory effects of β_2-microglobulin on ALP activity and calcification are shown in Figure 4. At a β_2-microglobulin concentration of $2\text{-}3x10^{-2}$mg/ml, which corresponds to the plasma β_2-microglobulin level found in dialysis patients (12), its inhibitory effect on calcification was detected while cell proliferation, collagen synthesis and ALP activity remaind unchanged. At the end of the 14 day culture in

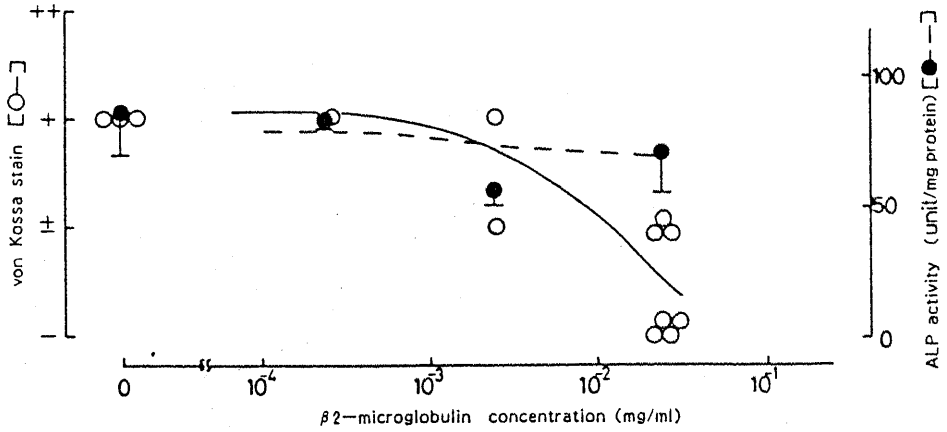

Figure 4. Effects of β_2-microglobulin on ALP activity and calcification in a cell culture assay.

the presence of β_2-microglobulin, 0.20±0.05 μg/dish of β_2-microglobulin was detected from the supernatant fluid obtained by collagenase treatment followed by sonication of cultured cells, while less than 0.003 μg/dish was observed as a control culture without the addition of β_2-microglobulin, which suggests a significant amount of β_2-microglobulin added was deposited in the cell layers.

DISCUSSION

Demonstration of aluminum deposition in various tissues, especially in bone, of dialysis patients (13) and the possibility of aluminum removal by administration of desferrioxamine (14) have led to a strong interest of the effects of aluminum on bone growth and mineralization. In this study, the effect of aluminum at concentrations comparable to those found in dialysis patients was evaluated on calcification in vitro. The results indicate that aluminum suppresses calcification without exerting any appreciable effect on proliferation, collagen synthesis or ALP activity, suggesting a significant role of aluminum in the prevention of hydroxyapatite formation in vivo. Thus, the pathogenicity of aluminum, which was suggested first in a clinical setting, was supported by in vitro findings in this

study. These findings further indicate the necessity of therapeutic modalities to decrease the presence of aluminum in the tissues of dialysis patients. For this purpose, the use of aluminum-free dialysate, the administration of non-aluminum containing phosphate binders, and a combined modality of desferrioxamine administration with dialysis particularly using a membrane with larger pore size (7) have been proposed and are now being evaluated.

On the other hand, in vitro findings on aluminum obtained in this study demonstrate the validity of the bioassay model using the osteoblastic cells. With this model the inhibitory effect of β_2-microglobulin on in vitro calcification was also demonstrated in this study. In addition to detection of β_2-microglobulin in bone of a dialysis patient (3), this finding strongly suggests a role for β_2-microglobulin in the impaired bone metabolism in dialysis patients. Although β_2-microglobulin is not permeable to conventional dialysis membranes, a few non-cellulosic leaky membranes such as BK-membrane (7) not only permit β_2-microglobulin to pass through but have been shown to adsorb it. Detailed clinical trials for effective removal of β_2-microglobulin by using these membranes are under way at present.

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