Extracts of Porcine Pancreas Prevent Progression of Hypercalcemia and Cachexia and Prolong Survival in Nude Mice Bearing a Human Squamous Carcinoma

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ABSTRACT

Porcine pancreatic extracts (PXs) have previously been shown to decrease blood ionized calcium in BALB/c mice (T. Yoneda, Y. Takaoka, and G. R. Mundy, FEBS Lett., 278: 171—174, 1991). In the present study, we show that the PX is effective in preventing progression of hypercalcemia and decreasing osteoclastic bone resorption associated with a human squamous carcinoma in nude mice. PX inhibited osteoclast-like cell formation in mouse bone marrow cultures and bone resorption in organ cultures of fetal rat long bones which had been stimulated by serum-free culture supernatants of this cancer. In addition, PX increased food intake, decreased weight loss, and prevented development of cachexia. In parallel with these effects, PX prolonged survival of tumor-bearing animals. PX might have therapeutic potential for management of hypercalcemia and cachexia associated with malignancy.

INTRODUCTION

As originally described by Takaoka et al. (1), we recently observed that PX decreases Ca\(^\text{2+}\) in BALB/c mice and suggested that the hypocalcemic associated with acute pancreatitis might be due to the hypocalcemic action of the PX released from the pancreas in response to acute inflammation (2). Interestingly, we also found in the same experiments that mice given the PX showed an increased food intake (2). Although the mechanism by which PX promoted the appetite of mice was unclear, Takaoka et al. (3) also reported that the PX promoted the appetite and prolonged life span in mice with muscular dystrophy and suggested that the PX might have anabolic effects on protein metabolism. These findings prompted us to explore the therapeutic potential of PX for diseases associated with imbalance of mineral metabolism and nutrition. In the present study, we have examined the effects of partially purified PX on nude mice bearing a human squamous carcinoma (MH-85) which was derived from the tumor of the maxilla of a patient who manifested marked hypercalcemia and cachexia (4). MH-85 tumor-bearing nude mice developed hypercalcemia and cachexia with anorexia, which were also found in the patient from whom the tumor was derived (5). We have found that partially purified PX diminishes development of hypercalcemia and cachexia in MH-85 tumor-bearing nude mice. In addition, PX prolongs the life span of tumor-bearing animals.

MATERIALS AND METHODS

MH-85 Tumor and Cells. The MH-85 tumor was isolated from a human squamous cell carcinoma of the maxilla (4) and has been serially transplanted in nude mice (BALB/c-nu/nu, male, 4—6 weeks old; Harlan-Sprague-Dawley) for more than 5 years in our laboratory. As seen in the patient with this tumor, s.c. inoculation of MH-85 tumor fragments (approximately 3 x 3 x 3 mm) under the skin of the right dorsal region induced marked hypercalcemia with increased osteoclastic bone resorption and cachexia with anorexia and decrease in body weight in nude mice as the tumor grew (5). Determination of Ca\(^\text{2+}\), body weight, tumor size, and food consumption was carried out as described (5).

MH-85 cells were established in culture from the tumor formed in nude mice as described previously (6) and grown in a minimal essential medium (Hazelton Biologies, Inc., Lenexa, KS) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and 1% penicillin-streptomycin solution (GIBCO Laboratories, Grand Island, NY). Serum-free culture supernatants of MH-85 (MH-85 CM) were harvested from 48-h cultures of confluent MH-85 cells.

Porcine Pancreas Extract PX. Crude porcine pancreas extract was prepared as described (2). Supernatants of porcine pancreas homogenates in 0.1 m Tris-HCl (pH 8.0) containing 2% sodium chloride were fractionated with 30—60% ammonium sulfate. The precipitates were dissolved in distilled water, dialyzed, and lyophilized (crude PX). PX crude was subjected to a DEAE (Whatman Labsales, Hillsboro, OR) anion exchange column (2.5 x 90 cm; Kontes, Vineland, NJ) which was equilibrated with 0.1 m Tris-HCl buffer (pH 7.5) and eluted with a stepwise gradient of NaCl (20 ml/tube) at a flow rate of 120 ml/h. Active fractions which inhibited bone resorption that had been stimulated by 1,25D\(_3\) in fetal rat long bone assay were pooled, dialyzed against 50 mM ammonium bicarbonate, and applied to a Sephacryl S-200 HR (superfine; Pharmacia, Piscataway, NJ) column (2.5 x 90 cm; Kontes) and eluted (10 ml/tube) at a flow rate of 90 ml/h. Active fractions were pooled and lyophilized (partially purified PX).

Administration of Partially Purified PX into MH-85 Tumor-bearing Nude Mice. PX prepared as described above was dissolved in phosphate-buffered saline and injected i.p. once a day for 4—6 weeks using a 27-gauge needle into MH-85 tumor-bearing nude mice with hypercalcemia and cachexia starting at 6 weeks after tumor inoculation. All in vivo experiments were carried out twice except for the experiments shown in Figs. 3 and 9 and reproduced the similar results.

Assay for Bone Resorption and Osteoclast Formation. Bone resorption was assessed by determining 45Ca release from fetal rat long bones in organ culture as described (7). Bones were labeled with 50 μCi 45CaCl\(_2\) (ICN Radiochemicals, Costa Mesa, CA) by giving the mothers injections 1 day before removal of the fetuses. Long bones (radius and ulna) were dissected from 19-day-old fetal rats with removal of cartilage and connective tissue. Bones were cultured in 0.5 ml serum-free Fization-Jackson modified Biggers, Gwatkin, and Judah medium (Sigma Chemical Co., St. Louis, MO) supplemented with 1 mg/ml bovine serum albumin serum albumin. Bones were incubated on steel grids at the interphase between medium and a 95% air-5% CO\(_2\) atmosphere at 37°C. All bones were cultured in medium for 24 h to remove exchangeable 45Ca. Bones were then cultured for 120 h in BgJb medium supplemented with 1 mg/ml bovine serum albumin and factors to be tested. Medium was collected at days 2 and 5. At the end of culture, bones were placed in 50% trichloroacetic acid for 1 h and 45Ca radioactivity in medium and trichloroacetic acid extract of bones was measured in a liquid scintillation counter. Bone resorption was assessed as the percentage of total 45Ca that was released into the medium.

Osteoclast formation was assessed by counting the TRAP(+)MNC number in mouse bone marrow cultures according to the method previously described (8). Bone marrow cells harvested from 4—6-week-old male ICR Swiss mice (Harlan Industries, Houston, TX) were incubated on 24-well plates (2 × 10\(^5\) cells/0.5 ml/well) and cultured in a minimal essential medium supplemented...
with 10% fetal calf serum (Hyclone) in the presence of $10^{-8} \text{M} 1,25\text{D}_3$ or MH-85 CM with or without partially purified PX for 6 days. The cultures were washed in phosphate-buffered saline, fixed in 60% acetone in citrate buffer (pH 5.4), air dried, and stained for TRAP using a commercial kit (Sigma). All TRAP(+)MNCs (cells stained in red with more than 3 nuclei) were counted manually under light microscopy.

Statistical Analysis. All data were analyzed by analysis of variance followed by a paired t test. In the experiment shown in Fig. 9, the statistical difference of the survival rate between control and PX-treated animals was analyzed by Breslow modified nonparametric Kruskal-Wallis one-way analysis of variance (9).

RESULTS

Partial Purification of PX. A peak of activity was eluted from DE52 anion exchange column chromatography with 0.2 M NaCl where there was little detectable protein (Fig. 1b). This activity inhibited 1,25\text{D}_3-stimulated bone resorption as measured by $^{45}\text{Ca}$ release from fetal rat long bones. These fractions with 1,25\text{D}_3-stimulated bone resorption-inhibiting activity were pooled and next applied on a Sephacryl S-200 HR gel filtration column. The peak of 1,25\text{D}_3-stimulated bone resorption-inhibiting activity was eluted at an apparent molecular weight between 25 and 67 kDa (Fig. 2).

The potency of partially purified PX was compared with that of crude PX in vivo. Two $\mu$g of partially purified PX exerted hypocalcemic action in BALB/c mice to the degree equivalent to that of 1 mg of crude PX (data not shown).

Effects of Partially Purified PX on $\text{Ca}^{2+}$ and Bone in MH-85 Tumor-bearing Animals. Nude mice bearing MH-85 tumors showed a continuous increase in $\text{Ca}^{2+}$ 6 weeks after tumor inoculation (Fig. 3). At 6 weeks after tumor inoculation when animals manifested profound hypercalcemia (Fig. 3, day 0), injection of partially purified PX was initiated. Injection of the partially purified PX (2 $\mu$g/mouse/day) i.p. every day for 5 weeks prevented further progression of hypercalcemia, although this treatment did not normalize $\text{Ca}^{2+}$. Two hundred ng/mouse/day PX had no effect on $\text{Ca}^{2+}$ and 20 $\mu$g/mouse/day caused the death of mice (data not shown). The reason for this effect of high concentrations of PX is not known. The impurity of the PX might have caused such an effect. The hypercalcemia in MH-85-bearing nude mice is associated with histological evidence of increased osteoclastic bone resorption at the endosteal surface (Fig. 44) as we reported previously (5). In contrast, calvariae of MH-85 tumor-bearing nude mice treated with PX showed a marked decrease in osteoclast formation at endosteal surface of the calvariae of PX-treated mice (Fig. 4B). Furthermore, there was a prominent increase in osteoblastic activity resulting in new bone formation at endosteal surface of the calvariae of PX-treated mice. Although histomorphometrical analysis was not carried out, this histological observation suggests that PX may inhibit the differentiation of hematopoietic mononuclear cells to mature osteoclasts.

Effects of Partially Purified PX on Osteoclast Formation and Bone Resorption in Culture. In in vitro studies, MH-85 CM stimulated the formation of TRAP(+)MNC with osteoclast characteristics in mouse bone marrow cultures to a similar extent to 1,25\text{D}_3 (Fig. 5) and increased $^{45}\text{Ca}$ release from fetal rat long bones in organ culture (Fig. 6). Addition of 100 ng/ml partially purified PX to these cultures decreased MH-85-CM-stimulated TRAP(+)MNC formation (Fig. 5) and inhibited bone resorption (Fig. 6). The partially purified PX also decreased TRAP(+)MNC formation in 1,25\text{D}_3-treated cultures. These effects of partially purified PX were obtained in a dose-dependent manner (data not shown).

Effects of Partially Purified PX on MH-85 Tumor-bearing Animals. In parallel with the development of the hypercalcemia, MH-85-bearing nude mice showed a marked decrease in body weight compared with non-tumor-bearing animals (Fig. 7). This decrease was
Fig. 4. Histological examination of calvariae of MH-85 tumor-bearing animals treated with or without PX. Calvariae of animals studied in Fig. 3 were removed 1 day after the final injection of PX or phosphate-buffered saline from animals bearing MH-85 tumor, fixed in 10% formalin, and decalcified. Note the differences in osteoclastic bone resorption (arrows) between untreated (A) and PX-treated (B) MH-85 tumor-bearing animals. Calvariae of PX-treated animals (B) showed fewer osteoclasts, less irregular endosteal surfaces, and narrower bone marrow space due to new bone formation (stars) resulting from an increase in osteoblast number than those in untreated tumor-bearing animals. Ca^{2+} in untreated and PX-treated animals shown were 2.41 and 2.37 mmol/liter before the initiation of treatment and 2.38 and 1.82 mmol/liter at the time of sacrifice, respectively. H & E, × 80.

partly due to reduced food intake (Fig. 8). Daily i.p. injection of partially purified PX for 4 weeks (2 μg/mouse/day) resulted in an increase in food intake by MH-85-bearing animals (Fig. 8), which was accompanied by significant gain of body weight (Fig. 7). In parallel with these effects, PX increased the life of MH-85 tumor-bearing animals (Fig. 9). Non-tumor-bearing mice showed no change in body weight after PX as we reported earlier (2). The PX had no effect on tumor growth (data not shown). We also found that PX increased food intake, body weight, and life span in cachetic animals bearing another human squamous cancer, OKK (5), which was not associated with hypercalcemia (data not shown).

DISCUSSION

Hypercalcemia is one of the most common paraneoplastic syndromes which occur in patients with advanced cancer (10). In the present study, we have shown that partially purified porcine pancreas extract PX prevents progression of the hypercalcemia caused by the MH-85 tumor in nude mice. We already showed that increased osteoclastic bone resorption was prominent in hypercalcemic nude mice bearing MH-85 tumors (5) and have found the identical results here. In addition, we have shown that the PX decreased osteoclastic bone resorption in the calvariae of MH-85 tumor-bearing animals based on qualitative analysis of histological sections. The hypercalcemia and increased osteoclastic bone resorption in MH-85 tumor-bearing animals based on our previous results that PX inhibited bone resorption stimulated by...
parathyroid hormone, interleukin 1α, tumor necrosis factor, transforming growth factor α, 1,25D₃, and prostaglandin E₂ in fetal rat long bones in organ culture (2), are consistent with the notion that the effects of PX on hypercalcemia in MH-85 tumor-bearing nude mice are likely to be mediated through inhibition of osteoclastic bone resorption caused by a soluble product(s) of the MH-85 tumor. Our results suggest that PX can be a preventive or maintenance agent for treatment of the hypercalcemia of malignancy.

Another notable action of partially purified PX is that the PX increases the appetite of cachectic MH-85-bearing nude mice. Anorexia (loss of appetite) is one of the characteristic features of cancer-associated cachexia, which is associated with a profound loss of body fat and skeletal muscle (11). MH-85-bearing nude mice manifested severe cachexia with anorexia (5). PX reversed reduced food intake and increased body weight in MH-85-bearing nude mice. Recovery from anorexia by the PX might be due to alleviation of the hypercalcemia, since it is well recognized that hypercalcemia causes loss of appetite (10). However, we previously found that PX increased food intake in non-tumor-bearing, nonhypercalcemic normal BALB/c mice (2) and in cachectic but nonhypercalcemic tumor-bearing nude mice in the present study. Thus, although the mechanism by which PX increases the appetite of normal and cachectic tumor-bearing animals with or without hypercalcemia remains to be elucidated and awaits the identification of PX, our results demonstrate that the PX has appetite-promoting and weight-increasing actions and suggest that the PX might be a useful therapeutic agent in cachectic cancer patients with anorexia and profound weight loss. This action together with prevention of serum calcium elevations in patients liable to hypercalcemia might be beneficial in some patients with advanced cancer.
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REFERENCES

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