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Alkaline Phosphatase in Serum and Tumour of Rats Bearing a Hormone-Responsive Transplantable Osteogenic Sarcoma*

P. M. INGLETON, L. A. COULTON, C. J. PRESTON and T. J. MARTIN

Department of Zoology and Chemical Pathology, University of Sheffield S10 2TN, United Kingdom

Abstract—*Serum alkaline phosphatase (AP) levels in rats bearing a hormone-responsive transplantable osteogenic sarcoma correlate closely with the growth of the tumour. Loss of serum AP activity after tumour removal together with data on heat susceptibility of tumour and serum AP provide strong circumstantial evidence that the serum AP is derived from the tumour and is similar to bone isoenzyme. Increased levels of the enzyme were not detectable until the tumour had reached a readily palpable size.*

Tumour AP production was unaffected by castration or ovariectomy but thyro-parathyroidectomy reduced tumour growth slightly and tumour levels of AP, whilst increasing serum AP as a function of tumour size. A single acute injection of salmon calcitonin into tumour-bearing thyro-parathyroidectomised rats significantly reduced serum AP activity. The tumours of such treated rats had significantly higher AP activity than tumours of similar rats treated with bovine parathyroid hormone.

INTRODUCTION

SERUM alkaline phosphatase (AP) activity, which is comprised of enzymes originating mainly from liver, bone, intestine and kidney, is elevated in various disease states. Alkaline phosphatase in bone is associated with the activity of differentiated osteoblasts, and osteogenic sarcomata classified histologically as osteoblastic are particularly rich in the enzyme [1]. The transplantable osteogenic sarcoma used in the experiments to be described in this paper was induced originally by injections of ^{32}P-orthophosphate [2]. The tumour is composed mainly of osteoblast-like cells capable of laying down ground substance which can mineralize [3]. The tumour cells have a hormone-responsive adenylate cyclase which responds to parathyroid hormone (PTH) and to prostaglandins E_1 and E_2. Isolated tumour cells also respond, to a small extent, to calcitonin (CT), indicating either that the tumour consists of two cell types or of single cell type which is responsive to both PTH and CT [4]. Since this transplantable tumour is composed mainly of cells with osteoblastic properties and AP is associated with the activity of such cells, the present experiments were carried out to determine whether any relationship existed between tumour growth and the level of activity of the enzyme in serum.

Various hormonal factors are known to affect AP activity. Parathyroid hormone decreases AP activity in isolated bone cells [5], and tissues sensitive to androgenic and estrogenic hormones show an increase in AP activity after treatment with these hormones [6]. In man osteogenic sarcomata occur most frequently in the second decade of life and more frequently in males than females [7, 8], a phenomenon which may be related to sex steroid production. It is possible that the AP activity of the osteogenic sarcoma may respond to differences in the circulating levels of parathyroid hormone, calcitonin, estrogens and androgens. Therefore, serum AP activity was measured in rats bearing the transplantable osteogenic sarcoma in which the hormonal milieu was altered by castration, ovariectomy or thyro-parathyroidectomy.

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MATERIALS AND METHODS

Osteogenic sarcoma

The osteogenic sarcoma has been main-

tained continuously in the same strain of inbred Sprague–Dawley rats in which it was originally induced [3, 9]. It has now undergone serial transplantation at approximately 3–4 weekly intervals for 3 yr, and has maintained its hormone-responsiveness throughout this period [3, 4]. For routine implantation, tumour slurry was prepared by pressing a small piece of tissue through 60 gauge stainless steel mesh, with the addition of Hanks' balanced salt solution, approximately 15 ml/10 g of tissue. Tumour suspension was injected subcutaneously into the left flank of recipient rats, 0.5 ml per rat (approximately 10^7 cells); sterile conditions were maintained throughout.

Determination of alkaline phosphatase activity

Alkaline phosphatase activity was measured in 10 μl samples of serum, in triplicate, by addition of 0.5 ml of 1.2 nM *p*-nitrophenol phosphate in 0.1 M diethanolamine buffer, pH 9.8 [10]. After incubation for 10–30 min at 37°C the reaction was stopped by addition of 2 ml of 0.05 M sodium hydroxide and the concentration of *p*-nitrophenol was read by absorption at 410 nm. The results are expressed as U/l (μ mole substrate cleaved/min/l serum). AP activity of tumour tissue was similarly measured in homogenates made in distilled water and the results expressed as activity per gram tissue per minute.

Surgical procedures

All surgical procedures were carried out under ether anaesthesia. Male and female rats were 6 weeks old when either ovariectomized or castrated, and subsequently received tumour implants 2–3 weeks later; sham-operated intact animals were similarly treated.

Complete bilateral thyro-parathyroidectomy was performed on 7 week old male rats, which were subsequently maintained on thyroxine supplemented (100 ng/ml) drinking water (TPTX rats). Three days after the operation serum calcium was measured in blood samples collected from the tail vein after an overnight fast. Serum calcium of 6 mg/100 ml was the upper limit accepted in TPTX rats. Readings were made on a Corning Calcium Analyser 940. The rats received a tumour implant 9 days after thyro-parathyroidectomy. Similar sham-operated intact rats served as controls.

Blood samples were always collected from the tail vein under light ether anaesthesia and tumour removal from the subcutaneous implant site was carried out under aseptic conditions, using ether anaesthesia.

RESULTS

Serum AP concentration in relation to tumour growth

It was found in preliminary experiments that rats with established osteogenic sarcoma had elevated levels of serum AP. Therefore, to determine the changes in serum AP during tumour growth, blood samples were collected from rats throughout the period of tumour growth. Tumour was implanted as previously described in 6 female rats and serum samples were collected at intervals until the rats were sacrificed 21 days after implantation when the tumours were very large. Each time a serum sample was collected the tumour volume was calculated by multiplication of readings, taken with calipers, along three axes of the tumour at right angles to each other. The results of this experiment are shown in Fig. 1. Serum

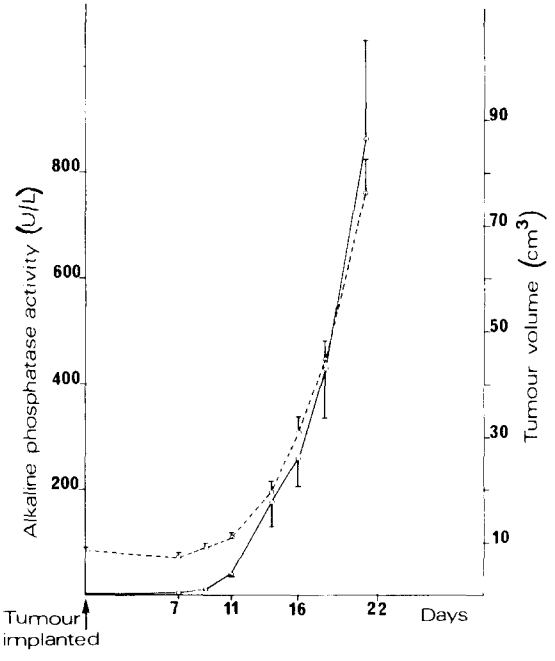

Fig. 1. Tumour volume (——○——) and serum alkaline phosphatase (- - -○- - -) in rats bearing a transplanted osteogenic sarcoma, measured at intervals during tumour growth. (n = 6), one S.E.M. shown at each point.

AP at tumour implantation was the basal level for these intact female rats (Fig. 1). There was no significant increase in the level of serum AP activity until 11 days post-implantation, although by this time the tumours had attained a reasonable size (4.11 $\pm 0.83\ cm^3$). Subsequently serum levels of AP activity showed a continuous and sustained rise which correlated closely with the increase in size of the tumour ($r = 0.99$). Neither the level of enzyme activity nor the tumour volume reached a plateau before the rats were sacrificed.

Identification of the source of the increased serum AP activity in tumour-bearing rats

(a) *Effect of tumour removal and tumour recurrence on the level of serum AP activity.* When osteosarcoma tissue was removed surgically from each of 4 female rats 17 days after implantation, and serum samples taken at intervals thereafter, there was a considerable fall in the serum AP activity in each animal (Fig. 2), suggesting that the elevated serum

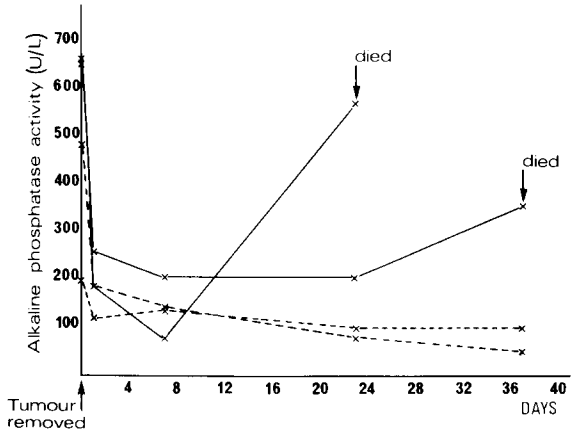

Fig. 2. Alkaline phosphatase activity in serum of each of four rats from which a subcutaneous osteogenic sarcoma was removed 17 days *after implantation.*

AP activity was derived from the tumour. The rats were bled subsequently at infrequent intervals, in two of the rats, which developed recurrent tumours at the original implantation site, the serum AP activity rose again (see Fig. 2). There were no obvious tumour metastases in these rats. In a further experiment serum samples were collected from two rats during eight hours immediately following removal of a tumour implanted 15 days previously: within 4 hr the serum AP activity had fallen to 50% of the initial level and remained at that level subsequently. In two sham-operated, intact, non-tumour-bearing rats serum AP activity also fell slightly during the first 3 hr post-operation, but then rose during the following three hours to almost original levels.

(b) *Heat susceptibility of alkaline phosphatase.* Further evidence that the serum AP was derived from the osteogenic sarcoma, and that the AP enzyme was similar to bone isoenzyme, was obtained by exploiting differences in heat stability of the AP isoenzymes. Posen *et al.* [11] have shown that the isoenzyme from intestine is more stable to heat than that of liver which in turn is more stable than bone isoenzyme. To compare the heat stability of the serum and tumour AP with that of other tissues, crude homogenates of rat liver, intestine, newborn calvaria and tumour were prepared in distilled water and incubated at 56°C for 40 min; samples were removed for assay at intervals during this period. The results are shown in Fig. 3 which demonstrates that the rate of degradation of the

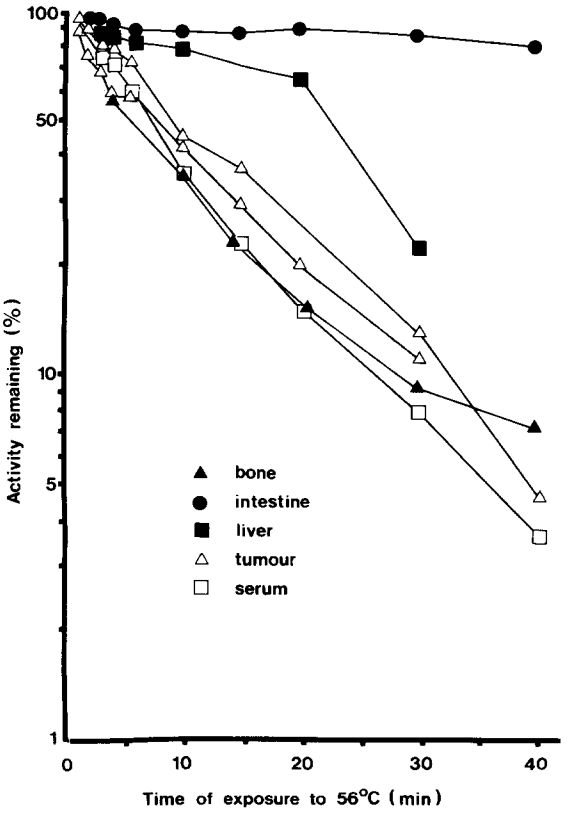

Fig. 3. Heat susceptibility of alkaline phosphatase of serum and tumour of oesteogenic sarcoma-bearing rats, and of normal rat liver, intestine and bone. Tissue homogenates and serum were incubated at 56°C *for* 40 min; 10 µl *samples of incubate were removed at intervals for estimation of alkaline phosphatase activity.*

tumour enzyme closely resembles that of bone and of tumour-bearing rat serum, and is much more rapid than that of either intestine or liver AP.

Effect of castration and ovariectomy on serum AP activity in rats bearing an osteogenic sarcoma

Tumour was implanted in the usual manner into male and female rats which had been castrated or ovariectomized. Serum AP activity was measured at the time of tumour implantation and at intervals during tumour growth for comparison with that of sham-operated controls. The results are shown in Table 1. The initial level of serum AP activity in the castrated males and in ovariectomized

Table 1. Serum alkaline phosphatase activity in rats receiving an implant of osteogenic sarcoma

Group	Alkaline phosphatase in serum (U/l) Mean ± S.E.M. Initial activity* (*n*)	Final activity† (*n*)
Intact female	159 ± 7.0 (6)	550 ± 60 (3)
	$P < 0.01$	NS
Ovariectomised female	126 ± 3.0 (6)	539 ± 127 (6)
Intact male	147 ± 6.8 (7)	970 ± 144.8 (7)
	$P < 0.005$	NS
Castrated male	122 ± 2.3 (9)	1105 ± 140.3 (8)
Intact male	147 ± 6.8 (7)	970 ± 144.8 (7)
	NS	NS
TPTX male	127 ± 12.6 (5)	749 ± 179.6 (5)

*Serum collected at the time of tumour implantation.
†Serum collected at sacrifice, 20 days after tumour implantation.

females was significantly lower than in their controls; however, the pattern of change in serum AP activity during tumour growth was similar in all groups, and there was ultimately no significant difference between serum levels in tumour-bearing castrated or ovariectomized rats compared with their intact controls. Thus the enzyme produced by the tumour did not appear to be responsive to changes in the circulating levels of sex steroids.

Effect of thyro-parathyroidectomy on serum AP activity in rats bearing osteogenic sarcomata

Parathyroid hormone (PTH) has been shown to decrease AP activity in isolated bone cells [5]. Isolated cells of this osteogenic sarcoma respond to PTH and calcitonin [4] with increases in cyclic AMP production. Therefore, the absence of these hormones from rats implanted with this osteogenic sarcoma may modify the AP activity of the tumour. Tumours were implanted into TPTX rats in the usual way and serum samples collected at intervals during tumour growth for comparison with sham-operated intact controls. Although initial and final levels of serum AP activity were lower in the TPTX rats than in controls, the differences were not significant (Table 1). However, the level of AP activity in the serum expressed per gram of tumour, was higher in the TPTX rats at sacrifice than in the controls (Table 2). Since the tumour at sacrifice represented a smaller percentage of the total carcass weight in the TPTX group of rats the results suggested a greater release of AP into the circulation in rats without PTH and calcitonin. The AP activity in the tumour tissue at sacrifice was lower in the TPTX rats than in the controls, which would also be consistent with the possibility that PTH and/or calcitonin may restrict enzyme release from the tumour cells (Table 2).

Table 2. Tumour weight and alkaline phosphatase (AP) activity in thyroparathyroidectomised (TPTX) osteogenic sarcoma-bearing rats at sacrifice*

Group	Tumour weight as % carcass weight. (mean ± S.E.M.)	Tumour AP activity per gram of tissue (mean ± S.E.M.)	Serum AP as a function of tumour weight (mean ± S.E.M.)†
Intact male (*n*)	21.2 ± 3.3 (7)	41.3 ± 3.4 (7)	19.0 ± 0.9 (7)
	NS	NS	$P < 0.05$
TPTX male (*n*)	13.1 ± 3.9 (5)	34.0 ± 2.1 (6)	31.4 ± 5.2 (5)

*Maintained on thyroxine supplemented drinking water (100 ng/ml).
†$\frac{\text{Alkaline phosphatase in serum (U/l)}}{\text{tumour weight in grams}}$

Action of parathyroid hormone (PTH) and salmon calcitonin (SCT) on serum AP activity in TPTX rats bearing a transplanted osteogenic sarcoma

Tumour was implanted into 15 TPTX rats; 22 days later when the tumours were large, the TPTX rats were divided into 3 groups of 5 rats; one group was injected subcutaneously with SCT (4700 U/mg, gift of Dr. J. W. Bastian, Armour Pharmaceutical Co., Kankakee, Illinois), 4.0 μg/rat, the second group received bovine PTH (1000 U/mg) 4.0 μg/rat, and the third group, was injected with vehicle only (3% cysteine hydrochloride with 0.1% bovine serum albumin). Serum samples were collected from the tail vein at the time of injection and subsequently 1, 2, 3, 4, 6 and 8 hr later. All three treatments produced a similar pattern of changes in serum AP activity, an initial fall followed by recovery (Fig. 4). However, during the first

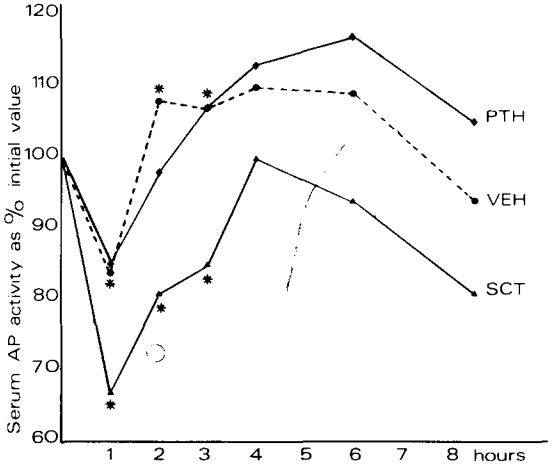

Fig. 4. Changes in serum alkaline phosphatase activity in thyro-parathyroidectomised rats bearing a transplanted osteogenic sarcoma after injection of bovine parathyroid hormone (PTH), salmon calcitonin (SCT) or solvent vehicle (VEH). For each group n = 5, * – * *VEH treated group and SCT treated groups differ significantly.* ($P < 0.05$).

3 hr after the injection there was a significantly greater reduction in the serum AP activity in the SCT treated group than in the vehicle treated rats; a result which is consistent with an inhibitory effect of SCT on AP release by the tumour.

The rats were killed 24 hr after the injection and the AP activity of the tumour was measured. The mean AP activity per gram of tissue was highest in the SCT treated group (171 ± 7), which was significantly higher than in the PTH treated group (142 ± 10, $P < 0.05$), but neither was significantly different from the group treated with vehicle (155 ± 10). Therefore, there was some evidence that SCT may have restricted loss or release of AP from the tumour cells in rats lacking endogenous PTH and calcitonin.

DISCUSSION

The results of these experiments on serum AP concentration in rats bearing a transplanted osteogenic sarcoma agree closely with those reported recently for transplantable murine osteosarcomata [12, 13]. In the mouse serum AP levels did not indicate the presence of small tumour masses but subsequent growth did correlate with serum AP activity. In one of these reports the mouse tumour size reached a plateau some 24 days after implantation [12] but in the present experiments rats with osteogenic sarcoma died before either serum AP or tumour size reached a plateau. The rat sarcomata were quite large before serum AP levels were significantly elevated, 11 days after implantation. There may be a number of possible explanations for the failure to detect an increase in serum AP before the tumour had reached a reasonable size. The rate of loss of enzyme from the cells may be low at this stage of development or the blood supply to the implant may be too poorly developed for enzyme to be taken into the circulation, and is instead broken down in the interstitial spaces. There is no significant variation in the concentration of AP in tumour tissue throughout the growth period (Coulton and Ingleton, unpublished observations). Thus monitoring of serum AP levels would not detect the presence of small, impalpable tumours. The close correlation between tumour size and the level of serum AP activity suggests that the enzyme is lost through "leaky" cell membranes or is normally released extracellularly, rather than representing the product of cell death. Normal bone growth in man, as measured by increase in body length, causes an increase in serum AP [14], so that enhanced release of the enzyme into the circulation is not necessarily associated with a disease condition but indicates increased activity of the bone cells. The *in vivo* half-life of the enzyme is short, as indicated by the rapid fall in serum levels after tumour removal, which suggests that there must be a continuous release of enzyme from the tumour at a considerable rate since serum levels represent a balance between accumulation and degradation.

The rapid fall in serum AP activity after tumour removal and its rise following re-

growth of the tumour, together with data on heat inactivation of the tumour AP all provide strong circumstantial evidence for the view that the high rate of serum AP activity in rats bearing a transplanted osteogenic sarcoma is derived from the tumour. The electrophoretic mobility of the tumour isoenzyme is similar to that of human bone (C. E. Wilde, personal communication) and its heat susceptibility is similar to that of rat bone AP; both these properties support the histological observations [3, 9] that the tumour is composed of osteoblast-like cells.

The insignificant effects of castration and ovariectomy on serum AP levels in rats bearing the sarcoma may indicate a genuine insensitivity of the tumour to the action of sex-steroids; on the other hand it may be that any *in vivo* inhibitory effects were masked by the presence of more potent stimulators. McMaster *et al.* [15] described experiments which demonstrated the presence of a factor in the serum of human osteosarcoma (OS) patients which stimulated the uptake of ^{3}H-thymidine into cultured human OS tumour cells, an effect which could be partially reduced by estrogens. It was shown some time ago by Li *et al.* [16] that growth hormone increased serum AP in hypophysectomized rats and in intact female rats. There are several factors in serum, including sulfation factors and thymidine-uptake factors which are dependent upon growth hormone for their production [17, 18] and which mediate the growth promoting actions of growth hormone. Thus an increase in thymidine-uptake activity in the serum may signify enhanced circulating levels of growth hormone which may stimulate production of tumour AP directly or indirectly. Such an effect could mask weaker inhibitory effects of other hormones. The role of the pituitary hormones in AP production by osteogenic sarcomata needs further investigation.

The osteogenic sarcomata in TPTX rats produced only slightly less AP into the serum during the later stages of tumour growth than did the intact controls. The tumours represented a smaller percentage of the carcass weight at sacrifice in the TPTX rats, which may account for the lower serum enzyme concentration, and the effects of the absence of PTH and calcitonin may have been abrogated to some extent by the presence of other stimulating agents. The higher serum AP levels in the TPTX rats in relation to tumour weight suggest that the tumour cells were losing the enzyme at an enhanced rate, and the PTH or calcitonin may be responsible for restricting this loss. *In vivo* injections of SCT produced a transient reduction in serum AP concentration and PTH failed to give a clear indication of reduced serum AP activity although the SCT-treated rats did have tumours containing a higher concentration of AP than those in PTH-treated rats. The action of these hormones may be more clearly demonstrated by *in vitro* experiments in the absence of other stimulatory or inhibitory factors.

The results of these experiments indicate that there may be various hormonal influences affecting the synthesis and release of alkaline phosphatase by this osteogenic sarcoma in the rat. Since there appear to be a number of factors affecting the growth of this tumour and its differentiated enzyme activity, *in vitro* techniques would appear to be the most useful for clarifying these effects and may help to throw light on the possible hormonal influences governing development, growth and activity of human osteosarcomata.

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