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Structural and Functional Correlations in Parathyroid Hormone Responsive Transplantable Osteogenic Sarcomas*

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Abstract—*Bone tumours induced in rats by repeated injections of* ^{32}P-*orthophosphate exhibit morphological characteristics of osteogenic differentiation after successive transplantation; stromal mineralization is a constant feature. Cell membranes isolated from the transplantable tumour possess a parathyroid hormone (PTH) and prostaglandin (PGE_1, PGE_2, $PGF_2\alpha$) responsive adenylate cyclase. Basal cyclic AMP (cAMP) concentrations and PTH responsiveness are greatest in the cellular poorly mineralized peripheral zones of the tumour. cAMP and PTH induce a cytoplasmic contraction response in cultured tumour cells and the PTH responsive adenylate cyclase is retained by the cells even after repeated subculture. A direct correlation exists between tumour size and serum alkaline phosphatase activity.*

INTRODUCTION

THERE are few model tumour systems in which well characterized biochemical properties of the neoplastic cells correlate with the structural and functional properties of the neoplastic tissue. We present in this paper our experience with a series of radiation induced osteogenic sarcomas, transplantable in the rat, that exhibit several stable functional and structural differentiated characteristics. Light and electron microscopic appearances, hormone responsiveness, alkaline phosphatase secretion and cultural properties are described. Some of the results are relevant to the apparent ambiguous relationship between cyclic nucleotide concentrations and neoplastic growth [1–3]. We also confirm the value of serum alkaline phosphatase activity as a possible marker for osteogenic neoplasia [4].

MATERIALS AND METHODS

Tumour induction and transplantation

Sprague–Dawley rats were injected intraperitoneally with carrier-free ^{32}P-orthophosphate according to a schedule of Bensted *et al.* [5]. An initial injection of 1 μCi/g body weight was followed, at 2 week intervals, by subsequent injections of 0.6 μCi/g body weight. Bone tumours appeared in roughly half of the animals, usually at the distal end of the femur, within 6–9 months.

For transplantation, small pieces of tumour tissue were forced through a 60-mesh stainless steel gauze and injected subcutaneously (s.c.) into the flank. Alternatively, cells were dispersed by digestion of tumour fragments with hyaluronidase and collagenase [6, 7] and injected (10^6–3×10^6 cells) into the flank.

Histological studies

Slices of tumour tissue were fixed either in Bouin's fluid or, if undecalcified sections were to be prepared, in neutral 10% formalin. Undecalcified sections were stained to demonstrate mineralisation by Von Kossa's method.

The relative volumetric composition of different zones within a tumour was derived from area measurements obtained by point counting tissue sections [8]. A regular 100 point grid lattice was mounted in the focal plane of the microscope eyepiece and the proportion of points coincident with the ima-

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ges of various tissue components was recorded for the entire section. Approximately 1000 points were assessed on each sample.

Transmission electron microscopy

Small fragments of tumour tissue were fixed in neutral phosphate buffered 3% glutaraldehyde, post-fixed in osmium tetroxide, and embedded in Araldite after dehydration with graded alcohols. Thin sections were stained with lead citrate and examined in an AEI Corinth transmission electron microscope.

Tissue cultures

Portions of fresh tumour tissue, macroscopically devoid of necrosis, were either cultured directly after mechanical mincing with scissors or after a further step of enzymic digestion with collagenase and trypsin in versene. Cultures were maintained in 25 cm^2 flasks using Waymouth's medium and 10% foetal calf serum. Subcultures were prepared by enzymic disaggregation of cell monolayers.

Scanning electron microscopy

Coverslip cultures were gently washed in cold buffered isotonic saline, fixed for 1 hr in cold neutral phosphate buffered 1% glutaraldehyde and washed in distilled water. After drying by the critical point method from liquid CO_2, and coating with gold and palladium under vacuum on an orbital jig, the cultures were examined in a Cambridge S4 surface scanning electron microscope at 45° to the electron beam.

Cyclic AMP content and adenylate cyclase activity

The cyclic AMP (cAMP) content of tumour tissue was measured in extracts by a competitive protein binding assay [9]. Hormone induced changes in adenylate cyclase activity in membrane preparations isolated from solid tumours or cultured tumour cells were studied as previously described [10].

Alkaline phosphatase

Alkaline phosphatase activity was assessed in serum by measuring the amount of phenol liberated from *p*-dinitrophenol as substrate [11].

RESULTS

Tumour morphology

Four ^{32}P-induced tumours were studied in detail. Most primary tumours resembled, in their overall architecture, spontaneous human osteogenic sarcomas with a bulbous expansion in the end of a long bone ensheathed by elevated periosteum.

After repeated transplantation the tumours displayed a stable morphological pattern characterized by monotonous cellularity with fusiform cells disposed in broad interlacing fascicles (Fig. 1). Centrally, coarse trabeculae of an osteoid-like material were present and this was usually associated with macroscopically overt calcification (Fig. 2). Undecalcified sections stained by von Kossa's method confirmed the presence of mineralization on the network of intercellular collagen that resembled osteoid (Fig. 3). On electron microscopy, coarse aggregates of deeply electron dense mineral were found to be associated with the intercellular matrix (Fig. 4).

Hormone responsiveness

The response of adenylate cyclase in membranes prepared from these tumours has been described previously [10, 12]. Parathyroid hormone (PTH), and the prostaglandins PGE_1, PGE_2 and $PGF_2\alpha$ stimulate adenylate cyclase activity in membranes and cAMP production in intact cells with the same relative potencies that these agents exert upon bone in organ culture [6, 7, 13]. The response of these tumours to hormones has not changed over a 3 yr period encompassing more than 30 transplantation passages.

Basal cAMP and PTH response in different regions of the same tumour

For this experiment portions of a single but representative tumour exhibiting different macroscopic features were taken and each divided into three parts for detailed biochemical and structural analysis. One part was fixed, processed for histology, and assessed for mitotic activity and volumetric composition by point counting. The second part of the specimen was immediately snap frozen in liquid nitrogen and the cAMP content assayed after deproteinization. PTH responsiveness was determined in cell membranes prepared from a third contiguous portion of tissue. The location of the sampled zones is shown diagramatically in Fig. 5 with the principal morphometric and biochemical data obtained.

Zones A (peripheral) and D (heavily mineralized) contained the least amount of necrotic tissue and also contained the most cAMP, 12.5 and 8.4 μmole/mg protein respectively (S.E. of method $\pm 5\%$). Although

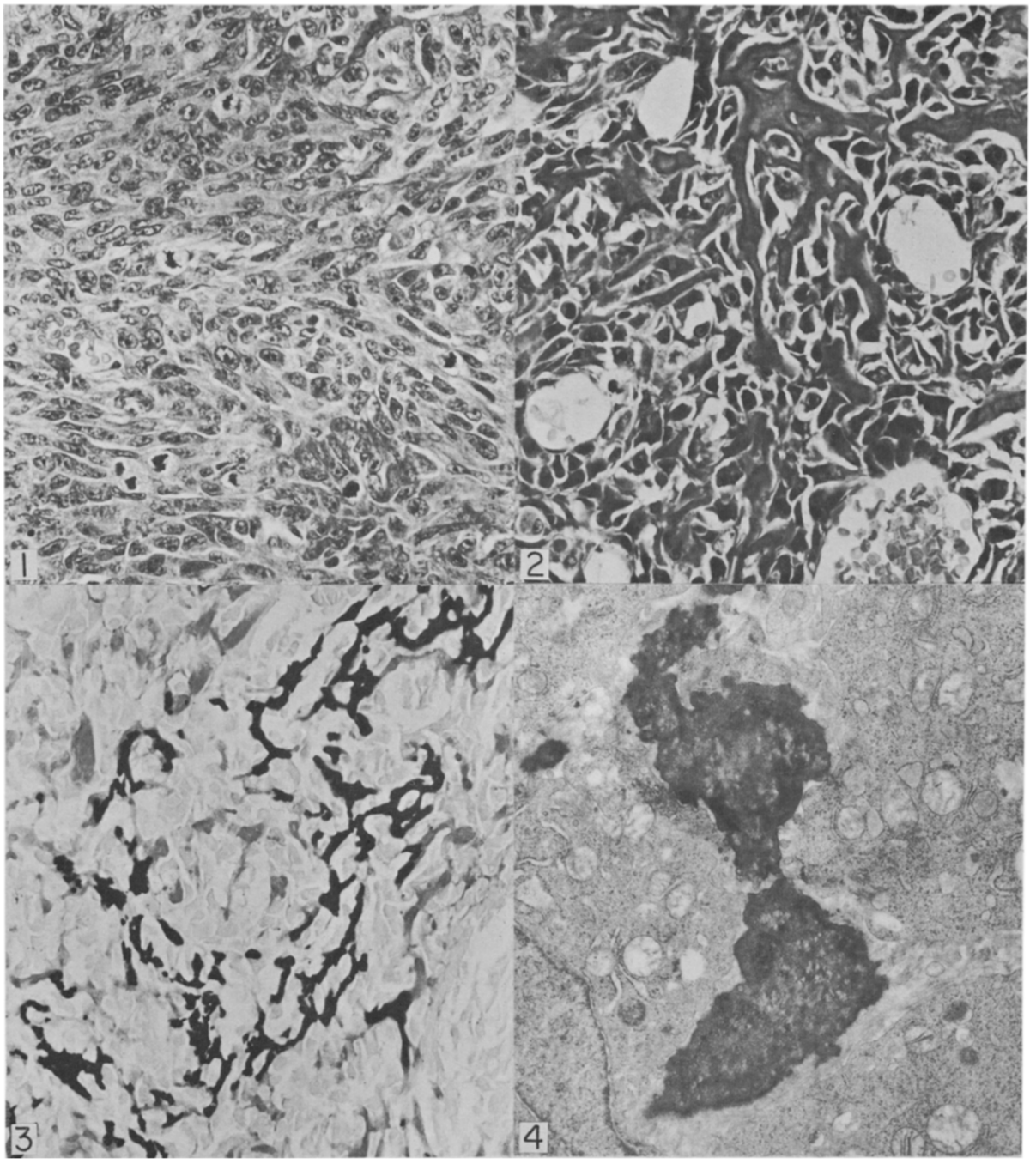

Fig. 1. *Cellular and mitotically active periphery of a transplanted osteogenic tumour after successive passages. Haematoxylin and eosin* (×310).

Fig. 2. *Central zone of a transplanted tumour. Osteogenic differentiation is evident from the presence of coarse trabeculae of intercellular matrix resembling osteoid. Haematoxylin and eosin* (×310).

Fig. 3. *Mineralization of intercellular matrix is shown by the dense staining superimposed on the weakly staining collagen. Undecalcified section—Von Kossa/Van Gieson* (×310).

Fig. 4. *Coarse electron dense mineral deposits are located in the intercellular matrix. Adjacent tumour cell cytoplasm filled with mitochondria, polyribosomes and profiles of rough endoplasmic reticulum. Transmission electron micrograph* (×7500).

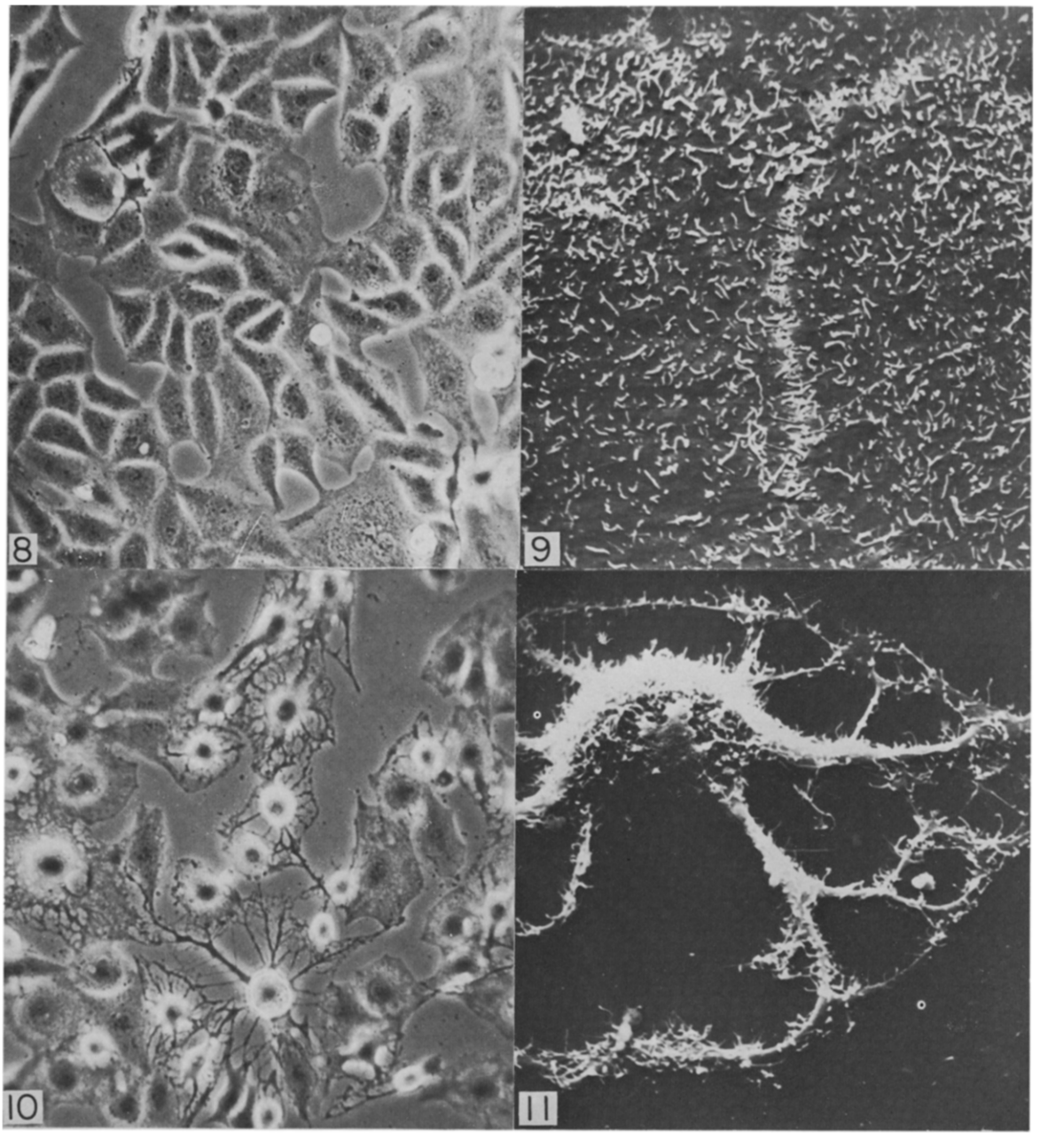

Fig. 8. *Osteogenic sarcoma cells from a transplanted tumour in culture. Phase contrast* (×200).

Fig. 9. *Osteogenic sacroma cells from a transplanted tumour in culture. The cell membranes bear numerous microvilli which appear to interdigitate at cell junctions. Scanning electron micrograph* (×2500).

Fig. 10. *Contraction of osteogenic sarcoma cells in culture* 90 min *after addition of* 1 mM *dibutyryl cAMP to the serum free medium. PTH elicited an identical response. Phase contrast* (×200).

Fig. 11. *Contracted osteogenic sarcoma cell under same conditions as in Fig.* 9. *Strands of cytoplasm radiate to the thin peripheral cytoplasmic rim. The microvillus configuration is unchanged. Scanning electron micrograph* (×2500).

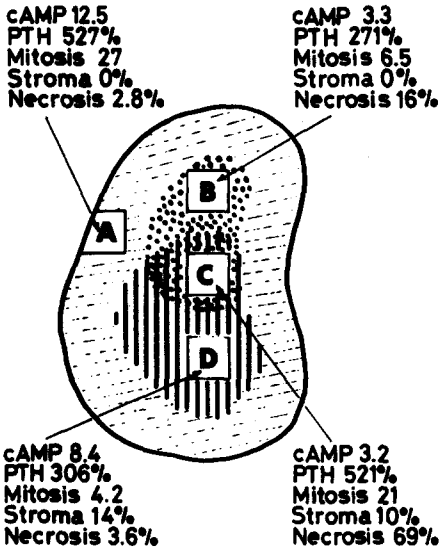

Fig. 5. Diagrammatic representation of the cut surface of the transplanted osteogenic sarcoma sampled for comprehensive parallel morphometric and biochemical analysis. Oblique light hatching—cellular periphery; stippled area—haemorrhagic necrosis; bold vertical hatching—macroscopic mineralization. Cyclic AMP content expressed as pmoles/mg *protein; PTH response as percentage increase in adenylate cyclase activity over basal activity; mitotic activity as mean number of mitoses per unit volume of viable tumour; stromal content and necrosis as percentage fraction of total tumour volume in that zone.*

zone B contained over 80% by volume of morphologically viable tumour tissue, the low incidence of mitosis (6.5 per unit volume of viable tumour) suggests that its overall viability was impaired; there was a reduced cAMP content of only 3.3 μmole/mg protein. Zones B and D showed the least mitotic activity (6.5 and 4.2 per unit volume of viable tumour) and had the least capacity to respond to PTH (271 and 306% increase over basal levels respectively).

Tumour growth and serum alkaline phosphatase

The sarcoma cells are rich in alkaline phosphatase. Following transplantation a marked rise in serum alkaline phosphatase activity occurs (Fig. 6) which correlates with the increase in tumour size as assessed by volume measurements (Fig. 7).

Properties of cultured tumour cells

Primary cultures were readily established from these tumours. The cells vary in shape from polygonal to bipolar (Fig. 8). Surface scanning electron microscopy disclosed that most cells had numerous surface microvilli with interdigitation of adjacent cell membranes at junctions (Fig. 9).

Adenylate cyclase response to PTH and PGE_2, at maximally stimulating concentration of 2×10^{-6} M and 10^{-4} M respectively, was retained in the cultured cells through at least ten subculture passages (Table 1). Moreover, it was invariably possible to store

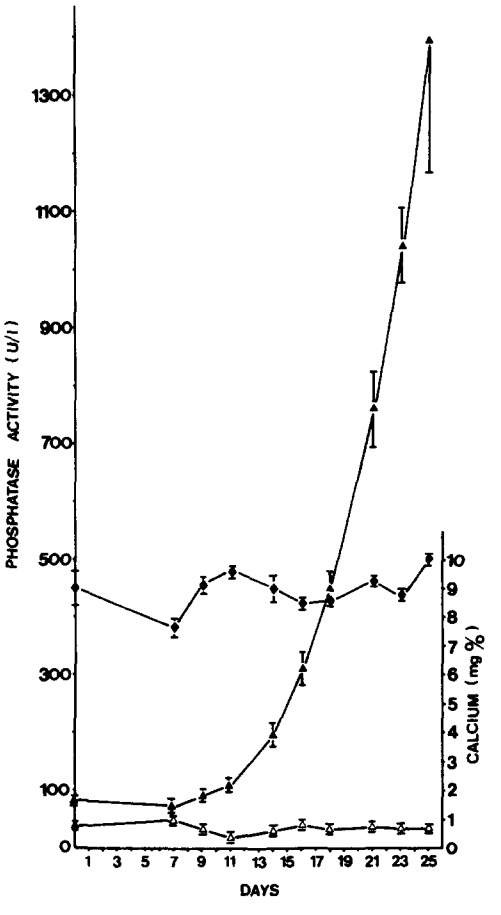

Fig. 6. Serum alkaline phosphatase activity plotted against time after transplantation. Osteogenic sarcoma bearing rats (▲); normal rats (△); serum calcium in osteogenic sarcoma bearing rats (◆), vertical bars indicate S.E.

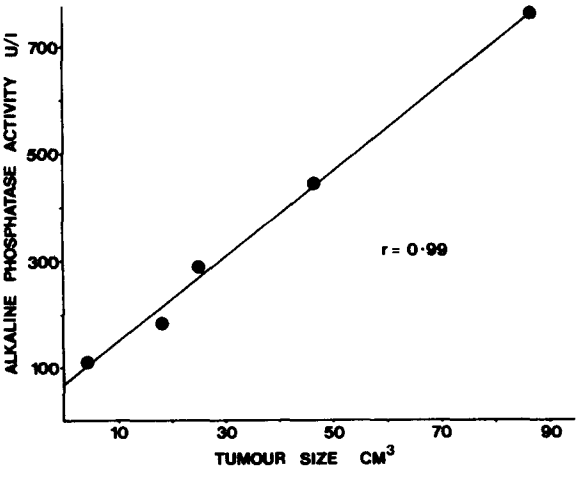

Fig. 7. Correlation between serum alkaline phosphatase activity and size of transplanted osteogenic sarcoma.

cells in liquid nitrogen with 10% dimethyl sulphoxide for several months and return them to culture with full retention of hormone responsiveness. On one isolated occasion, however, prolonged maintenance in culture under similar conditions resulted in loss of hormone responsiveness, but this was restored by a single transplantation cycle in the rat.

Table 1. Responsiveness of cultured osteogenic sarcoma cells to maximally stimulating hormone concentrations

Passage number	Percentage increase in adenylate cyclase activity over basal activity		
	PTH	PGE_2	NaF*
1	778±52	536±52	2394±257
2	1393±33	354±42	1900±133
5	1636±110	163±31	2400±273
8	985±61	239±8	2098±295
10	1297±156	534±19	2863±158

*NaF (Sodium fluoride) elicits maximal stimulation of adenylate cyclase activity.

Morphological changes induced by the addition of hormones or cyclic nucleotides to the medium were sought for in cultures maintained for the duration of the experiment in the absence of serum. The cells were placed in serum free medium 18 hr prior to additions. Cyclic AMP, as the more soluble dibutyryl analogue, (DbcAMP), at a concentration of 10^{-3} M consistently evoked cytoplasmic contraction. Sodium butyrate at a similar molar concentration had no effect. The first sign of contraction was visible within 30 min and was maximal at 90 min (Fig. 10). The contracted cells showed no alteration in the configuration of the surface microvilli (Fig. 11). The addition of PTH at a concentration of 3×10^{-8} M also produced cytoplasmic contraction morphologically indistinguishable from that elicited by DbcAMP. In a typical experiment, in which the percentage of maximally contracted cells of a total of 500 in each duplicate coded culture was scored visually, the results were 4.8±1.0% contraction in 10^{-3} M DbcAMP and 9.8±2.4% contraction in 2.5 ng/ml PTH, as compared to 2.2 ±2.4% contraction in control flasks. The solvent vehicle for PTH, 0.01 M acetic acid, had no effect.

DISCUSSION

Correlative studies of the biochemical and structural properties of malignant neoplasms are difficult unless, as in the case of these osteogenic sarcomas, there are easily identifiable biochemical and structural features. These osteogenic tumours provide additional information on the stability of functional characteristics in cell cultures and the potential use of alkaline phosphatase as a tumour marker substance for osteogenic neoplasia.

Transplantable tumours derived from spontaneous osteogenic sarcomas are well described [14, 15] although few continue to elaborate a mineralized stroma [16–18]. The administration of radioactive materials, notably radium or phosphorous, appears to be a reliable induction method for osteogenic tumours that consistently retain osteogenic differentiation [5, 19–21].

The osteogenic ability of these tumours was associated with the expression of responsiveness, to PTH and prostaglandins, for which receptors exist in normal bone. However, when different regions of a single neoplasm were critically examined there was no obvious relationship between mineral content and PTH responsiveness. The highly cellular, poorly mineralized peripheral zone was just as responsive to PTH as the more central heavily mineralized region. The central regions presumably represent mature parts of the neoplasm that have had more time to accumulate detectable mineralized matrix; the actual rate of matrix synthesis and mineralization may well be the same in both central and peripheral zones. Similarly, the roughly 4-fold variation in cyclic AMP content between different regions of a single tumour, emphasises the problems of drawing general conclusions about the relationship between tissue cyclic AMP concentration and the growth rate of a heterogenous neoplasm. This does not preclude the possibility that endocrine manipulation could be used to alter the overall cyclic AMP concentration within a hormone responsive tumour and, thus, modify its behaviour. Our preliminary attempts to do this have, however, not been successful.

The recognition of stromal mineralization in these tumours led us to seek the possibility that there might be elevation of serum alkaline phosphatase. The results show a clear cut linear relationship between tumour size and serum alkaline phosphatase and affirm the possible value of this enzyme as a tumour marker for human osteogenic sarcoma. Our results in this respect are similar to those obtained from the murine osteosarcoma model recently described by Ghanta *et al.* [4]. Further evaluation is necessary to determine

its usefulness in the monitoring of the human disease [22].

Tissue cultures can be reliably established from these radiation induced osteogenic tumours. The transient morphological changes in cultures of cells derived from normal rat bone by the addition of hormones and cyclic nucleotides [23] prompted us to examine the behaviour of the osteogenic sarcoma cells in the same way. As in the normal bone cell cultures, dibutyryl cAMP and PTH elicited a brisk cytoplasmic contraction response. Not all cells responded; this may reflect the heterogeneity of the cell population within the sarcoma cultures. The tendency of the cells to contract spontaneously was minimized by preincubation for 18 hr in serum free medium [23].

The very occasional loss of hormone responsiveness in prolonged culture, restored on retransplantation, has been previously demonstrated in other systems [24] including rat bone cells [25]. There are several possible explanations, such as the emergence of an unresponsive clone or overgrowth of the cultures by an unresponsive subpopulation. This must be a labile state because responsiveness was restored on transplantation.

The histogenesis of the predominant neoplastic cell in these tumours is speculative. The tumour cells form a bone-like ground substance and mineralize it, are rich in alkaline phosphatase activity, show little evidence of lysosomal activity and are chiefly mononuclear. These properties might indicate that the tumour cells are of osteoblast lineage. The pattern of hormone responsiveness, however, closely resembles that shown for bone resorption in organ culture [7, 13, 26, 27] a function which might be ascribed to osteoclasts or osteocytes. It is possible that the osteogenic sarcoma cell is akin to an osteoprogenitor [28] or primitive bone cell which has some of the features of bone resorbing cells (PTH and prostaglandin receptors) and of bone forming cells (matrix formation and mineralization, high alkaline phosphatase content). Present views on the origin of bone cells is, though, unsettled and some contend that osteoblasts and osteoclasts differentiate independently from mesenchyme [29].

These tumours will be valuable in determining hormonal influences on neoplastic growth and differentiation. Further attempts to characterise the lineage of the bone cells in these sarcomas may well help in understanding the sequence of events in bone cell differentiation.

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