Short Communication

Hypercalcaemia and in vitro osteolysis associated with xenografts of squamous carcinomas of the tongue

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It was previously reported that squamous carcinomas from several sites in the head and neck resorb bone in vitro by activating osteoclasts (Tsao et al., 1981). Preliminary bioassays indicated that these tumours contained increased amounts of "prostaglandin-like materials" (Bennett et al., 1980) and subsequent work confirmed that a mixture of indomethacin-sensitive prostaglandins (mainly E₂) and non-prostaglandin osteolysins was released by squamous carcinomas and their associated stroma (Tsao et al., 1981; 1983, submitted for publication). Xenografts have been grown from some of our established squamous carcinoma cell lines (Easty et al., 1981a, b) and this report describes the effects of such xenografts on plasma calcium levels in the host and their osteolytic activity in vitro.

The xenografts were grown from 2 cell lines from squamous carcinomas of the tongue: Cell line LICR/HN 5 was derived from a primary tumour and LICR/HN 6Rt from a locally recurrent lesion—see Easty et al. (1981a, b). The recipients were female mice of an inbred strain CBA/CaOla (Olac 1976 Ltd), immunosuppressed according to the method of Miller et al. (1963) with some modifications (Steel et al., 1978). Tumour xenografts were implanted s.c. into the flanks and passaged as solid fragments. Growth of the xenografts was followed by weekly measurement of external tumour diameters with vernier callipers. The wt of each animal was recorded and small samples (100–200 µl) of blood were collected from the orbital venous plexus.

Plasma calcium levels were determined in duplicate with a Corning 940 calcium analyser and, in later experiments, on a Technicon Auto-analyser II together with inorganic phosphate, alkaline phosphatase and urea. Albumin levels were determined manually using the bromcresol green method.

Bone resorption assays were based on the standard procedure described by Reynolds (1968). ⁴⁵Ca-labelled calvaria were dissected from neonatal mice (5–7 days) and cultured in vitro. Paired half-calvaria were used in the assay and bone resorptive activity was measured by the release of radioactive ⁴⁵Ca from the prelabelled bone into the culture medium during a 3 day incubation period. The in vitro osteolytic activity of the xenografts was examined by culturing tumour fragments in modified Bigger's medium supplemented with 5% heat inactivated rabbit serum and antibiotics (Tsao et al., 1981). Cell-free supernatants after 3 days incubation were prepared by filtration (0.45 µm Millipore filter) and used as test medium in bone cultures. The osteolytic activity was expressed in the usual way as a ratio of release of ⁴⁵Ca from labelled bone in test and control cultures. Four pairs of test and control bone cultures were used in each determination. Prostaglandins present in culture media were purified by thin-layer chromatography (Eastman & Dowsett, 1976) and quantitated by radio-immunoassay using two antisera raised separately against PGE₂ and PGF₂α from Steranti laboratories and ³H-labelled PGE₂ (160 Ci mM⁻¹) and PGF₂α (180 Ci mM⁻¹) from Amersham International Ltd.

The growth rate of the 2 xenografts was consistent, and transplantation was usually carried out at 6–8 week intervals when the tumour measured ~1.5 cm in diameter. Both grafts formed circumscribed nodules and never showed signs of local invasion or dissemination. Their histological features remained closely similar to those of the 2 squamous carcinomas from which they were derived. Serial estimations of plasma calcium (Corning 940 calcium analyser) were made and clear differences emerged between animals whose grafts grew progressively compared with those where the tumours failed to develop. Initial results from 10 tumour-implanted mice are shown in Figures 1 and 2. With both LICR/HN 5 (Figure 1) and LICR/HN 6Rt (Figure 2) progressively growing tumours were associated (after about one month) with parallel rises in plasma calcium levels. No changes in plasma calcium were observed in mice whose grafts did not grow.

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More plasma was collected from additional mice with progressively growing tumours and from ungrafted control mice and estimations of calcium, inorganic phosphate, alkaline phosphatase, albumin and urea were made (Table I). Hypercalcaemia was again confirmed in animals with progressively growing xenografts using another procedure (Technicon Autoanalyser II instead of the Corning 940 calcium analyser); inorganic phosphate levels tended to be low. Concentrations of alkaline phosphatase, albumin and urea were similar in both groups of animals. The parathyroid glands were checked in all mice at necropsy. No glandular enlargement and no histological evidence of hyperparathyroidism was observed in tumour-bearing animals. Immunoreactive parathyroid hormone levels were not measured.

The possibility that the hypercalcaemia might be due to prostaglandins released by the tumours was examined by investigating the potential blocking effects of indomethacin. Separate experiments were made in which the drug was given orally to mice with established LICR/HN5 tumours and intraperitoneally to mice with established LICR/HN5 and LICR/HN6Rt tumours, using doses of 5 mg in 100 ml drinking water and 0.5 mg ml\(^{-1}\) in 0.2 ml physiological saline respectively. No significant effects were seen: the tumours grew and plasma calcium levels continued to rise.

In vitro bone resorbing activities of the two xenografts are shown in Table II. The results are expressed conventionally as the ratio of \(^{45}\)Ca release by test and control cultures (Reynolds, 1968; Tsao
Table I Effects of growth of two xenografted tumours (LICR/HN 5 & LICR/HN 6Rt) on the levels of plasma calcium, inorganic phosphate, urea, albumin and alkaline phosphatase in host animals

| Xenograft tumour | Mouse no. | Mean tumour diameter (mm) | Calcium (mg 100 ml\(^{-1}\)) | Inorganic phosphate (g l\(^{-1}\)) | Urea (mg 100 ml\(^{-1}\)) | Albumin (g l\(^{-1}\)) | Alkaline phosphatase (iu l\(^{-1}\)) |
|------------------|-----------|--------------------------|-------------------------------|----------------------------------|--------------------------|-------------------------|---------------------------------|
| LICR/HN 5 (passage 7) | 1 + 2 | 19.8 | 13.5 | 3.4 | 38.4 | 24.0 | 112 |
|                   | 3 + 4 | 19.6 | 12.8 | 3.2 | 34.8 | 23.0 | 91 |
|                   | 5 + 6 | 17.3 | 12.5 | 3.9 | 27.0 | 24.0 | 93 |
|                   | 7 + 8 | 19.3 | 11.1 | 3.8 | 27.0 | 24.0 | 97 |
| LICR/HN 6Rt (passage 7) | 9 + 10 | 19.8 | 11.6 | 3.4 | 31.0 | 21.0 | 75 |
| Control (no tumour) | 11 + 12 | 9.3 | 4.7 | 33.0 | 24.0 | 102 |
|                   | 13 + 14 | 9.0 | 5.9 | 34.0 | 23.0 | 100 |

The plasma from pairs of mice with tumour grafts of similar size was pooled for these estimations.

Table II In vitro bone resorption by freshly excised xenografted tumours

| Xenografted tumour | Passage number | \(^{45}\)Ca release: (Test/control) | \(\Delta \text{pH}\) |
|--------------------|---------------|----------------------------------|----------------|
| LICR/HN 5          | 8             | \(2.22\pm0.4\)                   | 0.20 |
| LICR/HN 5          | 9             | \(1.82\pm0.07\)                  | 0.13 |
| LICR/HN 6Rt        | 8             | \(2.37\pm0.18\)                  | 0.08 |
| LICR/HN 6Rt        | 9             | \(2.36\pm0.11\)                  | 0.12 |

\(\Delta \text{pH} = \text{pH (control medium)} - \text{pH (test medium)}\)

Each test/control ratio is mean ± s.e. of 4 pairs of test and control cultures.

...et al., 1981). Osteolysis was not significantly inhibited by adding indomethacin (1 \(\mu\)g ml\(^{-1}\)) to duplicate cultures. Radioimmunoassay of tumour-conditioned media revealed only small amounts of prostaglandins—both PGE\(_2\) and PGF\(_{2a}\) being <5 ng ml\(^{-1}\).

Two xenografted squamous carcinomas of the tongue have been shown to induce hypercalcaemia in their animal hosts and to resorb bone in vitro. The hypercalcaemia develops in association with progressively growing xenografts which have reached a size of \(~1\text{cm}^3\). Plasma calcium values then rise in parallel with increasing tumour size. The parathyroid glands are macroscopically normal. Spread of tumour into local or distant bone was never observed, and no gross abnormalities were seen in the skeleton at autopsy. Both xenografts are, however, osteolytic in vitro and the most likely source of the mobilized calcium is bone. The absence of both in vivo and in vitro effects of indomethacin and the detection of only small (<5 ng ml\(^{-1}\)) amounts of PGE\(_2\) and PGF\(_{2a}\) in vitro suggest that the stimuli for calcium release, both in the intact animal and in tissue culture, are non-prostaglandin in nature.

Hypercalcaemia in the absence of detectable skeletal metastases is a well-documented feature of several forms of malignant disease, and there is extensive evidence that some tumours elaborate osteolytic factors which stimulate distant bone resorption and cause hypercalcaemia. Two experimental neoplasms, the HSDM\(_1\) fibrosarcoma in mice and the VX2 squamous carcinoma in rabbits, release large amounts of prostaglandin E\(_2\) in vitro and induce a hypercalcaemia which is reduced by treatment with indomethacin (Tashjian et al., 1972; Voelkel et al., 1975). In contrast, Seyberth et al., (1980) found that the Walker carcinosarcoma 256 provoked hypercalcaemia in rats which was not lowered by indomethacin given at doses sufficient to inhibit systemic production of prostaglandin E\(_2\). Clinical observations indicate that inhibitors of prostaglandin synthesis such as indomethacin or aspirin are ineffective in the treatment of hypercalcaemia in most patients with cancer (Brereton et al., 1974; Seyberth et al., 1975; Powles et al., 1982); and Tashjian (1978; also Tashjian et al., 1982) has suggested that probably <10% of tumour-associated hypercalcaemias can be attributed to excessive production of prostaglandins in the tumour—by neoplastic cells and/or their associated (host) stroma. Non-prostaglandin hypercalcaemic agents have been proposed such as parathyroid hormone, "osteoclast..."
activating factor” and certain other chemically ill-defined products (Mundy et al., 1974a, b; Raisz et al., 1978; Martin & Atkins, 1979; Josse et al., 1981; Nimberg et al., 1982) but their role in the hypercalcaemias of malignant disease is unclear. Ectopic production of parathyroid hormones by non-endoctrine tumours is rare (Easty & Carter, 1980) and the part played by osteoclast activating factors and similar agents is likely to remain confused until their chemical structure is clarified, assays are available and they can be studied under in vivo conditions. The nature of the osteolyisin(s) described here is at present unknown.

In summary: s.c. xenografts of 2 squamous carcinomas of the tongue induce hypercalcaemia in immunosuppressed CBA/CaOla mice in the absence of detectable bone invasion. Both xenografts do, however, resorb bone in vitro. Neither the hypercalcaemia nor the in vitro osteolysis are inhibited by indomethacin and levels of prostaglandins released from xenografts into culture media are low (PGE₂, PGF₂α both < 5 ng ml⁻¹). The bone resorbing factor elaborated by these xenografts thus appears to be non-prostaglandin in nature.

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