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PRODUCTION OF AN INSULIN-LIKE GROWTH FACTOR BY OSTEOSARCOMA[1]

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To test the possibility that osteosarcoma cells produce their own growth factors, we measured levels of insulin and somatomedin C (SMC), an insulin-like growth factor, in culture media of two cell lines derived from patients with that disease. SMC but not insulin levels increased three- to ten-fold over a period of 7 days paralleling the increases in cell number. Production of SMC was inhibited by cycloheximide.

The insulin-like growth factors are polypeptides which are structurally similar to insulin but which do not cross-react with anti-insulin antibodies. Their biological effects also are similar to those of insulin; in addition they are potent mitogens and stimulate proteoglycan synthesis in cartilage in vitro and in vivo (1). IGFs are produced by a variety of normal and malignant cells (2-5). In the latter, it has been suggested that IGFs are part of an autocrine system in which they interact with specific receptors to stimulate growth of the cells which secreted them (6).

Osteosarcoma cells have been shown to multiply in vitro in response to exogenous conditioned medium containing IGFs (7). In addition, elevated plasma levels of SMC or IGF-I biological activity have been found in some patients with osteosarcoma (8). In this paper, we report that IGF-I can be produced in vitro by osteosarcoma.

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Abbreviations used in text: SMC (somatomedin C), IGF (insulin-like growth factor), ND (not done)

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METHODS

Cell Samples:

Two osteosarcoma cell lines, ATCC CRL 1423 (OSI) and 1427 (OSII) (gift of Dr. E. Yunis, Department of Pathology, Children's Hospital of Pittsburgh) were derived from human "classical" osteosarcomas and have been characterized previously (9,10). For each cell line, cells were plated (2 x 10^6/dish) into 7 pairs of 100 mm tissue culture dishes in 15 ml RPMI 1640 (MA Bioproducts, Walkersville, MD) with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 1% antibiotics. After 1, 4-7, 10, and 14 days of incubation at 37°C in 5% CO_2/95% air, duplicate plates were harvested. Supernatants were stored at -20°C for growth factor assays (see below). Adherent cells were trypsinized, washed twice in RPMI 1640 and the total number of cells/dish determined by trypan blue exclusion.

Conditioned Medium:

Cells were grown in 15 ml RPMI 1640-10% fetal calf serum in each of five 100 mm tissue culture dishes. When almost confluent, dishes were washed twice with serum-free medium, covered with 10 ml RPMI supplemented with glutamine and antibiotics, and incubated for 48 hours. Supernatants ("conditioned medium") then were frozen at -20°C for use in experiments (below). To ensure that SMC measured in the conditioned medium had been synthesized by the tumor cells, duplicate dishes were set up in the same manner but with the addition of cycloheximide (25 μg/ml, Sigma, St. Louis, MO), an inhibitor of protein synthesis.

Growth Factors and Determinations:

Insulin (11) and SMC (12) were measured by previously described radioimmunoassays. Intra-assay coefficient of variation for the SMC assay at the concentrations of SMC reported below was 4.8%. To determine whether SMC-binding proteins interfere with the quantitation of SMC, multiple samples of conditioned medium were assayed both before and after processing by acid-ethanol extraction (13). There were no significant differences in the concentration of SMC in conditioned medium as compared with acid-ethanol extracts of conditioned medium. Furthermore, serial dilutions of sample supernatants containing peak values of SMC exhibited parallelism to the SMC standard curve run in conditioned medium.

Effect of Human Growth Hormone on Growth and SMC Production:

For each of 3 concentrations of growth hormone (gift of National Pituitary Agency, Baltimore, MD) (50, 100, 200 ng/ml) as well as for a growth hormone-free control, cells (2 x 10^6/dish) were plated in 10 ml RPMI 1640-10% fetal calf serum in 5 pairs of 100 mm tissue culture dishes. One, 4, 5, 6, and 7 days later supernatants were collected from duplicate dishes in each group and SMC levels assayed. In addition, cells were trypsinized and the total number of viable cells per dish at each time point was determined by trypan blue exclusion.

Effect of Conditioned Medium on Growth:

Cells were plated (2 x 10^6/dish) in 6 pairs of 100 mm tissue culture dishes in 15 ml of RPMI-2.5% conditioned medium, RPMI-5% conditioned medium, RPMI-10% conditioned medium, RPMI-25% conditioned medium, RPMI-10% fetal calf serum, or RPMI alone. Duplicate plates from each group were removed after 1, 3, and 10 days of incubation. The total number of viable cells per dish at each time point was determined by trypan blue exclusion.

RESULTS

To determine whether SMC could be produced by osteosarcoma tumor cells, we looked at SMC levels in supernatants from each of two well-characterized osteosarcoma cell lines. Results of one representative assay using each cell line are shown in Table

TABLE 1

PRODUCTION OF SOMATOMEDIN C BY OSTEOSARCOMA†

	OSI		OSII	
Time (days)	Cells (mean #/dish)	SMC* (IU/ml)	Cells (mean #/dish)	SMC* (IU/ml)
1	1.8×10^6	0.017 ± 0.002	1.5×10^6	0.014 ± 0.001
4	1.2×10^7	0.017 ± 0.002	9.7×10^6	0.074 ± 0.005**
5	1.3×10^7	0.021 ± 0.003	1.0×10^7	0.094 ± 0.006**
6	1.5×10^7	0.035 ± 0.003**	1.1×10^7	0.110 ± 0.014**
7	1.5×10^7	0.048 ± 0.008**	1.3×10^7	0.120 ± 0.000**
10	3.4×10^6	0.027 ± 0.002	ND[2]	0.058 ± 0.007**
14	2.7×10^6	0.020 ± 0.002	3.2×10^6	0.045 ± 0.007**

† One representative assay.
* 10% fetal calf serum has 0.017 IU/ml SMC; values given as mean ± standard deviation.
** p = .01 compared with day 1.

I. Within 24 hours of plating, supernatants were found to contain a mean of 0.017 IU/ml and 0.014 IU/ml for OSI and OSII, respectively, levels comparable to those found in 10% fetal calf serum alone. Both SMC levels and viable cell numbers peaked on day 7 (p = .01, compared with day 1 samples), and both subsequently declined. Insulin levels did not reproducibly increase to a significant degree (data not shown). Three other experiments with each line produced similar results, although in one experiment with OSI, SMC levels and cell numbers peaked on day 4. Serum-free conditioned medium also had measurable SMC levels (0.042 ± 0.004 IU/ml for OSI and 0.170 ± 0.014 IU/ml for OSII) compared with RPMI in which osteosarcoma cells had not been incubated (<.005 IU/ml, p = .001). When conditioned medium was collected in the presence of cycloheximide, SMC levels again were <.005 IU/ml, an effect not attributable to cell death. Although production of SMC by some normal tissues *in vivo* is known to be controlled by growth hormone, the addition of single doses of physiologic and supra-physiologic concentrations of human growth hormone had no effect either on SMC production or rate of cell growth in this system (data not shown). In contrast to RPMI-10% fetal calf serum, conditioned medium failed to reproducibly support cell growth.

DISCUSSION

A number of reports have described the production of a variety of "growth factors" by sarcoma cells, including multiplication stimulating activity (5) and transforming growth factor (12) by human fibrosarcoma. A human osteosarcoma-derived cell line, 2T, has been shown to produce a mitogen similar to platelet derived growth factor

which is clearly different from the IGFs (7). The importance of these substances in vivo is not clear, but it has been suggested that they exert autocrine or paracrine activity, causing multiplication of the parent cell or adjacent tumor cells (6).

Our results suggest that IGF-I, or somatomedin C, but not insulin, should be added to the list of substances produced by osteosarcoma cells. Whether osteosarcoma in vivo, or other osteosarcoma-derived cell lines, also will produce SMC is not clear. However, that two randomly selected cell lines produced SMC suggests that this may be a more general finding. In one report which examined a different osteosarcoma cell line, supplementation of medium with exogenous IGF was shown to enhance growth (7). This observation might suggest that some osteosarcoma may not be able to produce their own IGFs, but yet have a growth requirement for these factors. Because these substances are a heterogeneous group of polypeptides, it also is unclear what the relative importance of each is to tumor growth. The inability of osteosarcoma cells to grow in the presence of conditioned medium without serum suggests at least that the somatomedin C present in conditioned medium is not sufficient for growth. Further experiments are needed to determine whether conditioned medium stimulates nucleic acid or protein synthesis, and whether osteosarcoma cells can specifically bind insulin-like growth factors.

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