Insulin-like growth factor I supports differentiation of cultured osteoblast-like cells

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Received 15 March 1984

Rat calvaria cells grown in culture for one week had properties of osteoblasts: a high content in alkaline phosphatase and a marked cyclic AMP response to parathyroid hormone (PTH). In short-term experiments, insulin-like growth factor I (IGF I) stimulated the incorporation of [^{14}C]glucose into glycogen. When IGF I was present in the medium during 6 days the cell number increased slightly and there was a substantial, disproportionate rise in alkaline phosphatase activity of the cultures. Thus, IGF I stimulates growth, and in addition, and in contrast to other growth factors, mainly enhances differentiation of osteoblasts.

Rat calvaria cell *Osteoblast differentiation* *Alkaline phosphatase* *Insulin-like growth factor I*

1. INTRODUCTION

Among many hormones and growth factors tested for their effects on bone formation in organ cultures of rat calvaria, only insulin and the somatomedins were found to stimulate bone collagen synthesis [1,2]. IGF I has been shown to promote growth in vivo [3]. We have reported that human IGF I and IGF II stimulate synthesis of DNA, RNA and glycogen in primary cultures of rat calvaria cells enriched in the osteoblastic cell type. IGF I is 5-times more potent than IGF II [4].

With respect to long-term effects on bone cells in primary culture, we were especially interested to find out whether IGF pimarily stimulated replication or whether it also affected an osteoblastic property (alkaline phosphatase as a marker of differentiation) of the cultured cells.

2. MATERIALS AND METHODS

2.1. Cell preparation and culture

Osteoblast-like bone cells were isolated by sequential digestion of newborn rat calvaria in collagenase as in [4] and plated at a density of 0.5 mio per dish (Falcon, 35 mm) in medium supplemented with fetal calf serum (FCS, 1%). The medium consisted of Ham's F 12 medium and MEM Alpha medium (both from Gibco) in a 1:1 mixture and contained 50 units/ml penicillin and 50 μg/ml streptomycin. On the day after plating (day 1), the cells were rinsed with serum-free medium and were given 1.5 ml of the same medium which contained human serum albumin (HSA, 1 g/l) instead of FCS. Media were changed on days 1 and 4. All determinations were carried out with confluent monolayers 7 days after the establishment of primary cultures, i.e., after 6 days of culture in serum-free medium. For the determination of cell number, cells were detached from the dishes with trypsin–EDTA and counted in a haemocytometer.

2.2. Growth factors

Insulin and pure IGF I (prep. I/3) were kindly supplied by Dr R.E. Humbel (Zürich). Rat serum was obtained by aortic puncture from adult rats as in [4].

Platelets were obtained from healthy donors, separated by Fenwal CS 3000, generously supplied by Dr Gmür (Zürich) [5].

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A crude preparation of platelet-derived growth factor (PDGF) was used; 10^{11} platelet, by definition 1 unit, were washed once in PBS, centrifuged at 2000 × *g* for 20 min at 4°C, dissolved in 10 ml PBS and lyzed by 5 cycles of freeze-thawing. After centrifugation at 20000 × *g* for 30 min at 4°C, the supernatant was heated to 56°C for 30 min, cooled to 4°C and centrifuged at 20000 × *g* for 30 min and dialyzed against F12 medium. The material derived from 1 unit contained 43 mg protein.

With respect to stimulation of [^{3}H]thymidine incorporation into DNA of cultured chick embryo or rat fibroblasts, 5 munits/ml of this material are equivalent to 10% FCS.

2.3. *Alkaline phosphatase*

Alkaline phosphatase activity was determined as in [4] by measuring the cleavage of *p*-nitrophenyl phosphate to *p*-nitrophenol at pH 10.2 in homogenates of calvaria cell cultures.

2.4. *Hormonal stimulation and measurement of cyclic AMP*

Hormonal stimulation and measurement of cyclic AMP were performed as in [4] with the exception that the buffer solution (Dulbecco's buffer with 1 g/l HSA) contained no theophylline. Cell layers were preincubated for 10 min at room temperature, then exposed for 5 min to parathyroid hormone (synthetic bovine PTH-(1–34), 6800 IU/mg, Beckman Instruments).

2.5. *Glycogen synthesis*

Glycogen synthesis from [^{14}C]glucose was measured as in [6] by exposing the cells grown for 1 week for 4 h to IGF I in the presence of 1 μCi D-[U-^{14}C]glucose (270 mCi/mmol, Amersham).

3. RESULTS

Calvaria cells plated at a density of 0.5 mio per dish in medium supplemented with 1% FCS and cultured for 6 days in serum-free medium grew well. Confluence was reached after 1 week of culture. These cultures were rich in alkaline phosphatase with an activity of 8.3 ± 0.4 μmol/10^6 cells per h (mean ± SE; $n = 6$, 3 experiments in duplicate). Moreover, parathyroid hormone markedly stimulated cyclic AMP in the cells (table 1). The cells were further characterized by their response to

Table 1

Cyclic AMP response to parathyroid hormone in cultured rat calvaria cells

Medium	Cyclic AMP (pmol/dish)
Control	1.3 ± 0.2
PTH (0.1 nM)	15.8 ± 3.4
PTH (1 nM)	44.8 ± 6.8
PTH (10 nM)	125.8 ± 16.1

Cells were cultured for 1 week and exposed in situ to parathyroid hormone for 5 min as described in section 2. Results represent the mean ± SE of 2 experiments carried out in duplicate, $n = 4$

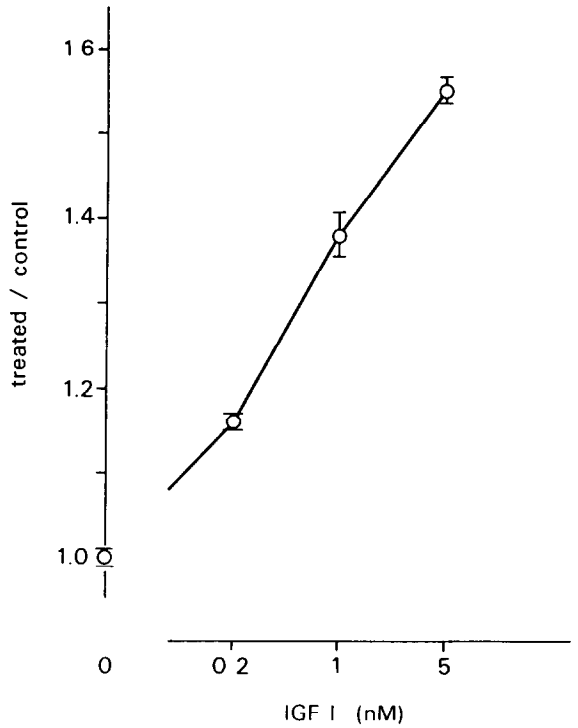

Fig.1. Stimulation of glycogen synthesis in cultured rat calvaria cells by IGF I. The cells were cultured for 1 week during 6 days in the absence of GCS or growth factors; 4 h before the end of the experiment, the medium was changed to one containing IGF I and [U-^{14}C]glucose (1 μCi/dish). Gycogen was isolated and counted as in [5]. Results from 2 experiments (4 and 5 dishes, respectively) are expressed as the ratio of the values of IGF-treated dishes over those of control dishes ($n = 9$, mean ± SE). The control values were 2489 and 2818 cpm/dish per 4 h, respectively.

IGF I with respect to glycogen synthesis [6]. They responded to IGF I with an increase of [^{14}C]glucose incorporation into glycogen (fig.1). In this respect they were at least as sensitive to the hormone as cells grown during the first 5 days in the presence of 5% FCS [6]. When IGF I was present in the medium during 6 days it tended to increase the cell number, and, at the same time, substantially raised alkaline phophatase activity of the cultures (fig.2) after 6 days of growth in defined medium. IGF I was found to be two orders of magnitude more potent than insulin; 100 nmol/l insulin under corresponding conditions increased the cell number by 12 ± 4% and alkaline phosphatase activity by 37 ± 5% (3 experiments, $n = 12$). Rat serum (0.5%) increased the cell number by 28% and inhibited alkaline phosphatase activity by 34% ($n = 3$). In fig.3, the effects of growth factors present in platelets and in serum are shown. Partially purified PDGF stimulates cell replication, and, at the same time, inhibits alkaline phosphatase activity in a dose-dependent manner.

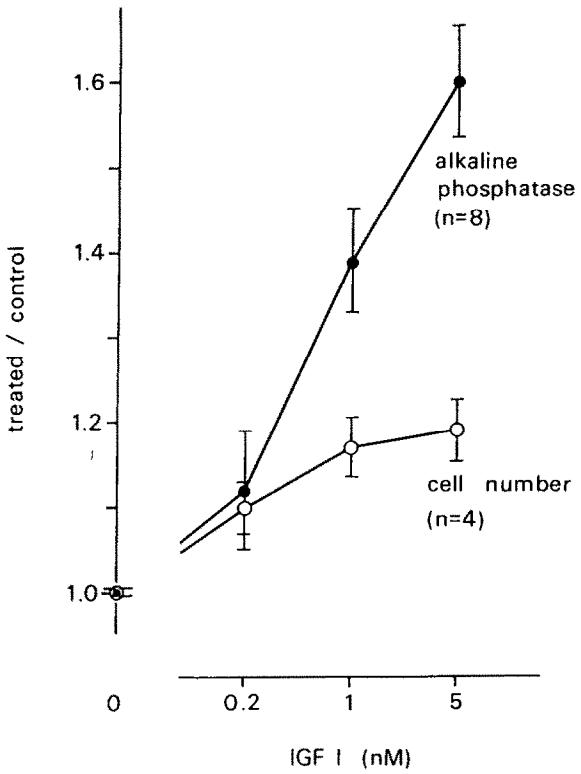

Fig.2. Cell number and alkaline phosphatase activity in primary cultures of rat calvaria cells grown for 6 days in the presence of insulin-like growth factor I (IGF I). After 1 week of culture, cell number and alkaline phosphatase activity were determined as described in section 2. Results from 2 experiments (2 dishes for cell counting and 4 dishes for determination of alkaline phosphatase activity) are expressed as the ratio of the values of IGF-treated dishes over those of control dishes; mean ± SE. The control values were 7.6×10^5 and 8.3×10^5 cells per dish for the cell number and 5.5 and 6.9 μmol/dish per h for alkaline phosphatase activity, respectively.

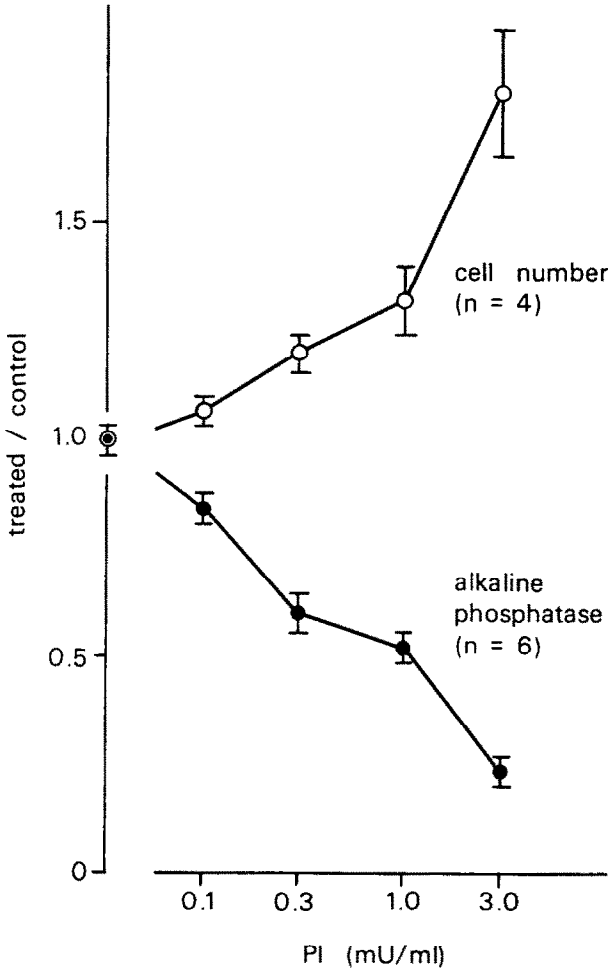

Fig.3. Cell number and alkaline phosphatase activity in primary cultures of rat calvaria cells grown for 6 days in the presence of crude PDGF (P1). Two experiments were carried out and their results plotted as described in legend to fig.2, mean ± SE.

4. DISCUSSION

Calvaria cells enriched in the osteoblastic cell type grow well in serum-supplemented media. Calvaria cells also replicate in the absence of serum, if seeded at a high cell concentration [7]. The cell density and concentration for plating in the present study (0.5 mio cells in 1.5 ml/35 mm diameter dish) were previously found to be critical for serum-free primary culture; the cells often failed to grow. We therefore included serum (1% FCS) in the medium to plate the cells and thereby improved the reproducibility of the culture conditions. Serum was replaced with albumin from day 1 to day 7 to permit a better observation of long-term effects of hormones on osteoblast-like cells in culture.

After 1 week, the cells had similar properties to identically prepared calvaria cells plated at a lower density and cultured in the presence of 5% FCS [4]. Alkaline phosphatase activity in the cultures was high, and the cells were quite sensitive to PTH: a concentration as low as 0.1 nM elicited a 12-fold rise in cellular cyclic AMP. The cells remained very sensitive to acute effects of IGF which enhanced the synthesis of glycogen from [^{14}C]glucose (fig.1).

IGF I is not very potent in stimulating DNA synthesis of serum-starved confluent rat calvaria cells [4]. The potency of hypox rat serum poor in IGF is similar to that of normal rat serum with regard to DNA synthesis [8]. It is, therefore, not surprising that IGF promoted cell replication only weakly, when compared to serum. On the other hand, IGF I clearly raised alkaline phosphatase activity of the cultures in a dose-dependent manner (fig.2). This is in keeping with the regulation of acute metabolic events. IGF alone and the IGF content of serum play a major role in the synthesis of glycogen [9]. Insulin was two orders of magnitude less potent, as reported with regard to short-term effects of the two hormones on the same cells [4]. It appears likely that the effects of insulin in supraphysiological concentrations are mediated by IGF receptors. It has recently been shown that insulin in rather large concentrations (100 nM) increased alkaline phosphatase activity in organ cultures of rat calvariae [10].

Platelet lysate containing PDGF as a potent mitogen depressed alkaline phosphatase activity (fig.3) and morphologically led to dedifferentiation of calvaria cells (not shown). In the organ culture model in [10] potent growth factors such as FGF and EGF also inhibited alkaline phosphatase activity [10]. Similar observations with EGF have been made with osteoblastic cell lines [11,12].

The role of alkaline phosphatase is poorly understood. A high content or activity of the enzyme is a characteristic property of osteoblasts in vivo and of calvaria cells maintained in vitro [13]. The finding that high concentrations of insulin and low concentrations of IGF I enhance alkaline phosphatase activity in primary cultures of osteoblast-like cells is in good agreement with the observation that IGF I and IGF II enhance muscle cell differentiation (myoblast fusion and a concomitant rise in acetylcholinesterase activity) in primary cultures of chicken embryonic cells [14]. IGF I, moreover, stimulates erythropoiesis [15]. Thus, the IGFs appear in a new light: they stimulate growth to some extent, but more importantly, they favour differentiation of cells of mesodermal origin.

ACKNOWLEDGEMENTS

We are grateful to R. Meister for her excellent secretarial work. This study was supported by the Swiss National Science Foundation (grants 3.380-0.78 and 3.167-0.81).

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