Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 142-147

GROWTH HORMONE DEPENDENT STIMULATION OF OSTEOBLAST-LIKE CELLS IN SERUM-FREE CULTURES VIA LOCAL SYNTHESIS OF INSULIN-LIKE GROWTH FACTOR I

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Received January 15, 1988

Gene-recombinant human growth hormone (rhGH) elicited a dose-dependent stimulation of the proliferation of osteoblast-like cells (OB), when grown in strictly serum-free longterm cultures. A half-maximal effect was observed at concentrations of 15-20ng/ml and the maximal stimulation was 160% of hormone-free controls. The rhGH-induced effect on proliferation could be inhibited dose-dependently by the addition of an insulin-like growth factor (IGF) I-antiserum to the medium. Moreover, IGF I and rhGH had additive effects only when the exogeneous IGF I concentration exceeded that of endogenously produced IGF I by a large margin. Thus, direct stimulation of OB proliferation by rhGH is, at least in part, mediated by IGF I-like immunoreactivity.

Stimulation of growth *in vivo* by growth hormone (GH) is easily demonstrable in GH-deficient animals (1,2). The mode of action of GH and the cellular mechanisms behind these effects are unclear. It is generally held that most of the effects of GH are indirect, i.e. mediated by GH-dependent plasma factors (2,3) - somatomedins or insulin-like growth factors (IGFs) - which are mainly produced by the liver (4). IGF I itself is a potent growth promoter of bone *in vivo* (5) and of chondrocytes and osteoblasts *in vitro* (6). To investigate whether GH has any direct effect on bone formation, we used an *in vitro* model with a population of enriched primary osteoblast-like cells (OB) under strictly serum-free conditions as a model. As previously shown (6), these cells express OB-characteristics such as high alkaline phosphatase activity and responsiveness to parathyroid hormone with respect to cAMP formation.

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Abbreviations: CP: collagenase digestible protein; (rh)GH (gene-recombinant human) growth hormone; (rh)IGF (gene-recombinant human) insulin-like growth factor; NCP non-collagen protein; OB osteoblast-like cell

0006-291X/88 $1.50

MATERIAL AND METHODS

Calvaria of newborn rats were sequentially digested with bacterial collagenase and the released OB were inoculated in serum-free viscous medium containing 0.8% methylcellulose as in (6). Various concentrations of gene-recombinant human IGF I (rhIGF I, courtesy of Drs. W.J.Rutter, San Francisco and J.Nuesch, Basle) and gene-recombinant human GH (rhGH, gift from Dr. L.Tofte, Nordisk) were added at the beginning of the culture period lasting 21 days. OB remain selectively as cuboidal cells and proliferate into cell clusters of clonal origin. The growth rate was monitored "on line" by counting the number of cells per colony on the same culture dish throughout the entire culture period. The average number of cells per colony was plotted as a function of time and the calculated slopes of linear regression lines ($r \geq 0.95$) describing the growth curves were compared.
The IgG-fraction of the anti human IGF I antiserum (kindly provided by Dr.K.Reber, Basel) which has been shown to crossreact with rat IGF I (4,7) was obtained by running both preimmune and immune serum over a protein A-sepharose column (Pharmacia). IgGs were eluted with glycine 0.1M/pH 3, dialyzed against PBS, diluted and filtered through a 0.22um Millipore filter.

For the determination of collagen synthesis, culture dishes (35 mm) were pulsed with 3uCi/ml 5-^{3}H-proline (20Ci/mmol, Amersham) in the presence of 50ug/ml ascorbic acid for 48 hrs at day 16 of culture. After labeling, 1ml of phosphate buffered saline containing 6.3mM N-ethylmaleimide, 2mM phenylmethylsulfonyl fluoride, 25mM EDTA, pH 7.2 (PBS^+) was added to the medium. The cells were scraped into the medium, collected and the dishes rinsed with 1ml PBS^+. After centrifugation, an aliquot of the cell pellet was used for protein determination and the remaining portion combined with an aliquot (1/10) of the medium to assay for ^{3}H-proline incorporation into collagen (CP) and noncollagen protein (NCP) using bacterial collagenase (Sigma, type VII) according to (8,9).

RESULTS

Addition of rhGH in physiological concentrations elicited a dose-dependent effect on cell proliferation of rat OB, grown in strictly serum-free medium. The minimal effective concentration of rhGH was 1ng/ml and half-maximal stimulation occured at 15-20ng/ml rhGH (Fig. 1). As observed in previous experiments (6,9), a basal growth rate of control cultures occurs even in the absence of any hormones. rhIGF I in physiological concentrations enhanced OB proliferation up to 250% of control cultures. Although both hormones stimulate OB replication in a dose-dependent fashion, the dose-response curves are not parallel and the hormone concentrations eliciting maximal effects differ considerably (Fig. 1, insert). When both rhIGF I and rhGH were present in the culture medium, the rate of cell proliferation increased to a greater extent than with the corresponding concentration of each hormone alone in parallel cultures. At maximal stimulation by rhGH (15ug/ml), OB proliferation was further increased only by high concentrations of rhIGF I (Fig.2). To test the possibility that GH might act through local production of IGF I by OB, we added the IgG-fraction of a neutralizing antibody against native huamn IGF I. This antibody cross-reacts with rat IGF I (4,7). The inhibition of the rhGH-induced OB proliferation was dependent on the dilution of the anti IGF I IgG-fraction, yet, basal growth was not inhibited (Fig.3). As a parameter of extracellular matrix synthesis, the ratio of ^{3}H-proline incorporation into CP and NCP was

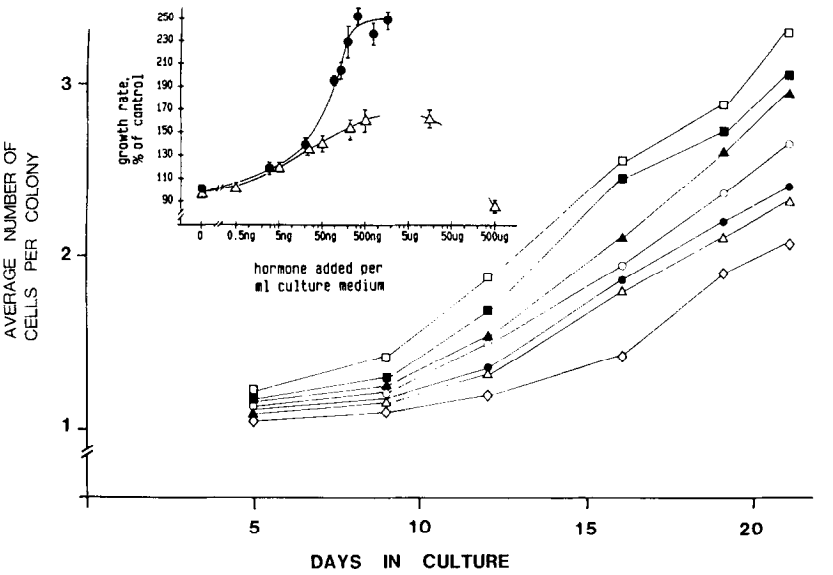

Fig.1. Effects of various concentrations of rhGH on the proliferation of OB prepared from newborn rats. rhGH was added at the beginning of the culture to yield final concentrations of ● 0.5ng/ml; ○ 5ng/ml; ▲ 50ng/ml; ■ 500ng/ml; □ 15ug/ml; ◇ 500ug/ml; △ control. **Insert** The growth curves were described by linear regression lines with correlation coefficients $r \geq 0.95$. The resulting slopes of these lines were compared to those obtained in the absence of hormones (100%). Each point represents the mean ± SEM of 3 proliferation assays (n=9) as outlined above derived from independent cell preparations. △ rhGH; ● rhIGF I

determined (Fig.4). rhGH at concentrations of 50ng/ml and more increased the relative content of collagen protein synthesis by about 100%. Human IGF I increased the relative collagen synthesis from 2.5% (control) to 9.2%

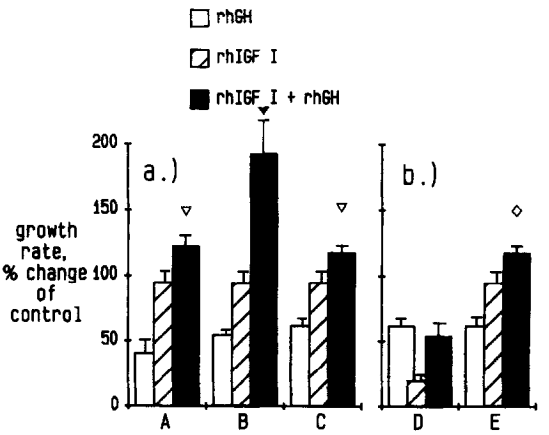

Fig.2. Effects of the combination of rhGH and rhIGF I on OB proliferation. Figures show slopes of regression lines as in Fig. 1. **a.)** Each dish contained 100ng/ml rhIGF I, plus 25ng/ml rhGH (A) or 250ng/ml rhGH (B) or 15ug/ml rhGH (C). **b.)** Each dish contained 15ug/ml rhGH, plus 3ng/ml rhIGF I (D) or 100ng/ml rhIGF I (E). Significantly different from cultures with 100ng/ml rhIGF I alone at ▽ $p<0.01$, ▼ $p<0.001$; from cultures with 15ug/ml rhGH alone at ◇ $p<0.01$
Mean ± SD, 2 experiments (n=6)

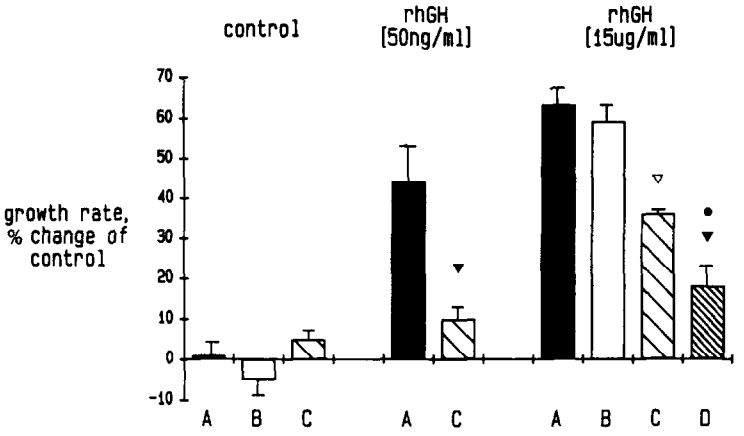

Fig.3. Inhibition of the rhGH induced OB proliferation by a rabbit anti human IGF I antibody. The IgG-fraction of the pre-immune serum (B, dilution 1:100) and of the IGF I antiserum (C, dilution 1:100; D, dilution 1:20) were added at the beginning of the culture period. Colony growth was compared to cultures containing the same concentration of rhGH but no IgG (A). Cell growth was monitored as in previous figures. Significantly different from (A) at ▼ $p<0.01$, ▽ $p<0.001$; from (C) at ● $p<0.01$.
Mean ± SD, 2 experiments (n=6)

(270ng/ml). Hence, the relation between the relative collagen synthesis upon exposure of OB to various concentrations of rhGH and IGF I are somewhat parallel to the growth curves (Fig.1) for the two hormones. Thus, we conclude that rhGH-induced proliferation is in part mediated by the local production of IGF I-like immuno-reactivity by cultured OB.

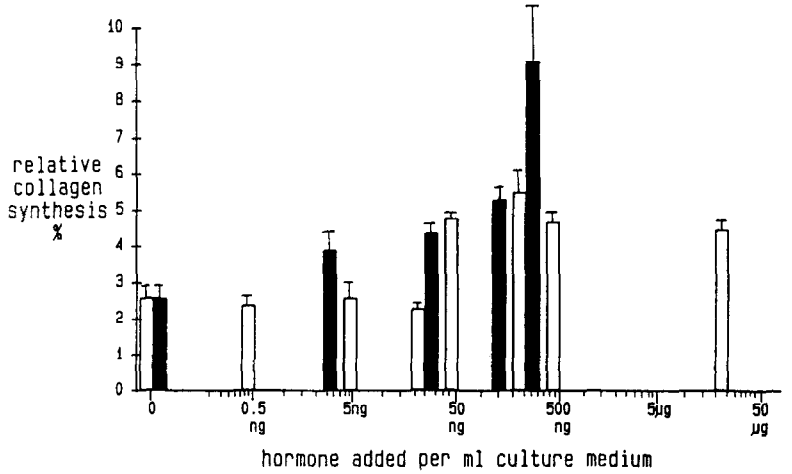

Fig.4. The relative amount of collagen protein synthesis was determined by ^{3}H-proline incorporation into CP and NCP as described in Material and Methods. Hormones at the indicated concentrations were added at the beginning of the culture. Open bars: rhGH; solid bars: rhIGF 1. Mean ± SEM, 3 experiments (n=6)

DISCUSSION

The present results demonstrate that GH alone stimulates cell proliferation of cultured rat OB which at no step of preparation or culture were exposed to serum, thus excluding the presence of any contaminating mitogen. Moreover, the use of gene-recombinant human GH excludes the possibility of a pituitary contaminant with mitogenic activity. A stimulatory effect was seen with rhGH concentrations within the range encountered in rat plasma (10). Furthermore, the results in Fig.3 provide evidence that rhGH-induced proliferation of OB involves the local synthesis of IGF I-like immunoreactivity. However, the IGF I antibody did not affect the basal growth rate of OB under serum-free culture conditions, indicating at least a second mechanism of autocrine/paracrine nature, different from IGF I.

So far, direct stimulatory effects of GH on OB have not been reported. Yet, GH exerts direct stimulatory effects on non-skeletal tissues such as hemopoietic progenitor cells (11), smooth muscle (12) and also on chondrocytes in concentrations of 50ng/ml or more (13). In short-term organ cultures of long bone, alkaline phosphatase activity, a bone specific marker is increased by GH (14), whereas the synthesis of collagen is not affected in rat calvaria (15).

Besides classical hormones, a variety of locally produced factors (16), part of which are IGF I-like (17), act also on bone. The latter may be synthesized in response to systemic hormones. GH has been shown to elevate IGF I-immunoreactivity in the medium of organ cultures of bone (14), which contain a substantial portion of non OB cells. These results do not permit to identify the population of cells responsible for the synthesis of IGF I-like immunoreactivity. On the other hand, several cell types release IGF I into the culture medium (18), suggesting that IGF I is not only a classical endocrine hormone. Local injection of GH into the growth plate causes an increased level of IGF I-immunoreactivity in the neighbouring cells (19).

If the effects of rhGH are mediated via locally produced IGF I (endogeneous), the maximal endogeneous IGF I concentration responsible for OB stimulation could be estimated by comparison with the dose-response curve of added rhIGF I (Fig.1, insert). In the presence of both hormones, rhIGF I would be expected to have additional effects on OB only if its concentration exceeded that of endogeneous IGF I by a large margin (Fig.2b). Thus, the combined effect of rhIGF I and rhGH may reflect the effect of total IGF I in the medium, consisting of locally produced endogeneous rat IGF I and added exogeneous rhIGF I (Fig.2a, lanes A, C). Yet, the combination of 250ng/ml rhGH and 100ng/ml rhIGF I leads to a greater effect than theoretically predicted (Fig.2a, lane B), pointing to an additional mechanism by which rhGH affects OB.

The bulk of organic bone matrix consists of type I collagen, and OB in vitro produce this type of collagen predominantly (>95%), as judged by polyacrylamide gelelectrophoresis (20; own unpublished observation). Yet, for reasons of accuracy, relative collagen synthesis was determined rather than absolute CP and NCP, since the protein recovery from dishes containing some 10^5 cells varied considerably. The qualitative effects of rhGH and rhIGF I on OB appear to be the same, since both hormones stimulate collagen synthesis and alkaline phosphatase activity (data not shown) in parallel to their effects on proliferation, thus fitting well into the concept of endogeneous IGF I synthesis by OB upon stimulation with rhGH.

Whether the same OB respond to both hormones at the same time or whether there may be a specific sequence of OB responsiveness to each hormone depending on the state of differentiation, as shown for a preadipocyte cell line (21), remains open.

ACKNOWLEDGMENTS

The authors thank Dr. Ch.Schmid for helpful criticism of the manuscript. This work was supported in part by Grant No. 3.051-084 from the Swiss National Science Foundation.

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