Mitogenic effects of growth hormone in cultured human fibroblasts. Evidence for action via local insulin-like growth factor I production.

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Research Article

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Mitogenic Effects of Growth Hormone in Cultured Human Fibroblasts
Evidence for Action via Local Insulin-like Growth Factor I Production

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Abstract

We examined human growth hormone's (hGH) effect on mitogenesis in cultured human fibroblasts, and the role of local insulin-like growth factor I (IGF-I). With 0.5% human hypopituitary serum (HPS), hGH increased thymidine incorporation (TI) over serum-free medium dose responsively, with half-maximal effect at 10 ng/ml (0.5 nM) (hGH 127±8.8%; IGF-I 107±1.7% [SEM]) ($n = 10$). Similarly, with 0.5% HPS, hGH and IGF-I increased cell replication by 172±8.2% and 169±25%, respectively ($n = 4$). Specific IGF-I monoclonal antibody (Sm1.2) dose dependently blunted TI stimulated by 10 ng/ml hGH or IGF-I (at 1:1000, 38±6.5% and 30±14% reduction, respectively). Sm1.2 also reduced cell replication by both 10 ng/ml hGH and IGF-I, respectively, to 32% and 42% of stimulated values. Dexamethasone (0.1 μM) synergistically enhanced TI by both IGF-I and hGH. A 28-h time course for TI showed that hGH stimulated a similar peak to IGF-I, lagging in its effect by 4–10 h. We have provided further evidence that hGH stimulates growth of cultured human fibroblasts via local IGF-I production, consistent with IGF-I's paracrine–autocrine role.

Introduction

Although it is well recognised that human growth hormone (hGH)[1] plays a central role in growth, the exact mechanism of its mitogenic effect in human tissues remain unclear, with evidence for direct and indirect actions.

In 1957 Salmon and Daughaday postulated that the growth-promoting effects of growth hormone (GH) are mediated via secondary substances, now known as somatomedins or insulin-like growth factors (1, 2). With the development of specific radioimmunoassays it became clear that insulin-like

growth factor I (IGF-I) levels correlated well with different growth states in humans and with GH levels (3). IGF-I may also play an important role in the negative feedback mechanism for hGH (4, 5). In vivo evidence for IGF-I's growth-promoting effect comes from infusion studies in hypophysectomised rats (6, 7). A paracrine and autocrine role for IGF-I is supported by the finding that it is produced by cultured human fibroblasts and acts locally on them (8–12). Clemmons and co-workers have provided indirect evidence that hGH acts via this local IGF-I production in cultured human fibroblasts. With frequent replacement of medium after GH treatment, DNA synthesis was blunted, suggesting action via a local product, possibly somatomedin-C (IGF-I), addition of which maintained DNA synthesis (10). However, a direct link between GH and IGF-I–mediated mitogenesis has not been established. Specific hGH binding sites have been demonstrated on cultured human fibroblasts so providing a potential site of action for hGH in this tissue (13).

There is evidence for GH exerting its mitogenic effect directly on target tissues, such as rat tibial cartilage (14, 15), erythroid precursor cells (16), and cultured rabbit chondrocytes (17). However, this does not exclude stimulation of local IGF-I production in these tissues. Nilsson et al. have recently demonstrated that GH injection in hypox rats stimulates local IGF-I synthesis in tibial growth cartilage (18).

Workers have failed to show an effect of hGH alone on DNA synthesis in fibroblasts (Rosenfeld, R. G., R. L. Hintz, personal communication), but recently the role of serum competence factors in cell growth has been emphasized (10, 19). These include platelet-derived growth factor, present in normal serum as well as in serum deficient in hGH and IGF-I from hypopituitary ("hypox") individuals (10). We therefore chose to examine the effects of hGH in the presence of small amounts of human hypox serum, and a cell line known to elaborate IGF-I locally. Our aims were to examine for possible mitogenic effects of hGH in cultured human fibroblasts and to further investigate whether hGH acts on fibroblasts via local IGF-I production.

Methods

Cell culture

Human fibroblasts from three cell lines cultured from the pubic skin of normal adult males were used. The cells were grown to confluency in tissue culture bottles (Corning Glass Works, Corning Science Products, Corning, NY) in 10% FCS-DME (Flow Laboratories, Inc., McLean, VA) between passages 6 and 15. They were plated in Linbro wells (1.6 cm diam; Flow Laboratories) at a density of 50–100,000 cells per 1-ml well. After being serum starved for 48 h, serum-free medium (SFM) or 0.5% hypopituitary serum (HPS, both containing 0.1% BSA; Miles Scientific Div., Miles Laboratories Inc., Naperville, IL) was added to the wells with or without hGH, IGF-I, and other additions.

1. *Abbreviations used in this paper:* Ab, antibody; DM, dexamethasone; EGF, epidermal growth factor; GH, growth hormone; hGH, human growth hormone; HPS, hypopituitary serum; IGF-I, insulin-like growth factor I; PDGF, platelet-derived growth factor; SFM, serum-free medium; TI, thymidine incorporation.

Materials

The HPS was obtained from a 20-yr-old male with undetectable serum levels of GH and IGF-I as measured, respectively, by radioimmunoassay (2nd antibody, polyethylene glycol; Royal Children's Hospital) and an Immunonuclear Corp. kit assay utilizing synthetic IGF-I, C_{18} Seppak extraction (sensitivity 5 ng/ml; cross-reactivity with human IGF-II < 1%). Final tissue culture concentrations of other pituitary-derived or -dependent hormones from this serum were as follows (all estimated by radioimmunoassay): cortisol 1.2 nM; thyroxine 0.33 nM; testosterone 0.01 nM; estradiol 0.05 pM; prolactin 1.3 mIU/liter. All of these concentrations are well below the physiologic range likely to contribute a mitogenic effect.

The IGF-I was obtained from Amgen Biologicals, Thousand Oaks, CA (ARN4050), prepared by an *Escherichia coli* host recombinant DNA procedure. Epidermal growth factor (EGF), tissue culture grade, was obtained from Seragen Inc., Boston, MA. Preliminary pilot studies were performed with pituitary-derived hGH, provided by the Australian Human Pituitary Advisory Committee, Canberra, Australia. All studies reported here were performed with highly purified synthetic methionine-hGH (Kabi Pharmaceuticals AB, Stockholm, Sweden) (Somatonorm III), prepared by an *E. coli* host recombinant procedure. The monoclonal antibody (Ab) to IGF-I (αSm1.20β) was kindly provided by Dr. L. E. Underwood, University of North Carolina at Chapel Hill, purified from mouse ascites fluid by precipitation with sodium sulphate, as previously described (20). It has ~ 30% cross-reactivity with IGF-II (Underwood, L. E., personal communication). However, IGF-II is not a known product of human skin fibroblasts and is only weakly mitogenic (21), so that such cross-reactivity is unlikely to be significant in these studies. A monoclonal heart worm Ab, prepared by a similar method and at a similar protein concentration, was provided by Dr. M. J. Waters, University of Queensland, Australia. Porcine Actrapid insulin was supplied by Novo Industri, Copenhagen, Denmark.

[³H]Thymidine incorporation (TI)

A similar method to that described by Conover et al. was used (19). Methyl-[³H]thymidine (5.0 Ci/mmol solution; Amersham International, Amersham, UK) was added at 21 h to give a concentration of 0.2 μCi/ml. At 24 h, the cells were washed three times with 1 ml of PBS on ice, incubated 5 min at 37°C with trypsin-versene, and harvested with a rubber policeman using 500 μl 10% trichloroacetic acid (TCA) per well, with a further wash of 500 μl of 10% TCA. The cells were harvested into borosilicate tubes and 25 μl FCS added to each tube before centrifugation at 3,000 rpm for 15 min and the pellet washed with 10% TCA. The pellet was solubilized overnight with protosol (0.5 M, New England Nuclear, Boston, MA). Aliquots of 100 μl were taken in duplicate and counted in the liquid scintillation counter using acidified phase combining system scintillant.

Cell replication

Cells were plated at a density of 40–70,000 cells per well in 1.6-cm Linbro wells. After being serum starved for 48 h, the cells were incubated for 4 d in SFM or 0.5% HPS with the experimental additions. The cells were then harvested after 5 min incubation with trypsin-versene at 37°C. After suspension in Osmosol, cells were counted in triplicate (Coulter Electronics Inc., Hialeah, FL).

Statistics

Statistical analysis was performed either by a program (Epistat) on a personal computer (IBM Instruments, Inc., IBM Corp., Danbury, CT) using the Student's t test for paired samples, or by the nonparametric Friedman two-way analysis of variance for comparison of multiple points.

Results

Stimulation of [³H]TI

The stimulation of TI in the fibroblasts by hGH and IGF-I is shown in Fig. 1. The data represent 10 experiments using a
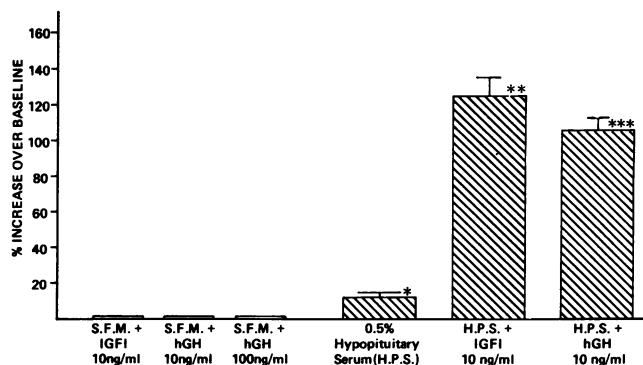

Figure 1. [³H]TI into human fibroblasts expressed as percentage increase over baseline counts for SFN (±SEM) (n = 10), *P < 0.01, **P < 0.001, and ***P < 0.001 relative to SFM.

single cell line. Due to baseline variations between individual experiments, the results are expressed as percentage increase over baseline counts for SFM. In the presence of SFM alone, neither IGF-I at 10 ng/ml nor hGH at 10 or 100 ng/ml gave an increase in TI. HPS (0.5%) gave a small mean increase in TI of 12±4% (SEM) (P < 0.01). The addtion of 0.5% HPS to IGF-I at 10 ng/ml gave a synergistic effect with a mean increase in TI of 127±8% (P < 0.001). Whereas hGH alone had no effect, in the presence of 0.5% HPS, hGH at 10 ng/ml similarly gave a mean increase in TI of 107±4% (P < 0.001). Subsequent experiments were all performed in the presence of 0.5% hypox serum, and in view of the consistently minimal effect of hypox serum alone, results were all expressed relative to serum-free controls. A dose response for TI stimulated by hGH at concentrations of 10, 50, 100, and 1,000 ng/ml was demonstrated (P < 0.01). The data represent four experiments using one cell line as shown in Fig. 2. The effect of hGH was dose dependent, with half-maximal effect in the physiologic range at ~ 10 ng/ml (~ 0.5 nM). In subsequent experiments hGH was therefore used at this half-maximal physiologic concentration, and compared with IGF-I at the same concentration. Since the effect of IGF-I on DNA synthesis is enhanced synergistically by dexamethasone (DM) (22), we examined the effect of DM on hGH-induced TI in these cultured fibroblasts. Fig. 3 shows the effect of addition of 0.1 μM DM to either IGF-I or hGH in the presence of 0.5% HPS. An almost threefold increase in TI was seen when 0.1 μM DM was added to IGF-I at 10 ng/ml (P < 0.01). A threefold increase in TI stimulated by hGH was similarly seen when 0.1 μM DM was added (P < 0.01).

To examine the possible role of local IGF-I production in the hGH-induced DNA synthesis, we examined the effect of concurrent addition of a specific monoclonal Ab to IGF-I. The monoclonal Ab to IGF-I was added at a dilution of 1:1000 (n = 8) (Fig. 4). The Ab reduced TI stimulated by IGF-I at 10 ng/ml (plus 0.5% HPS) by 30±14% (P < 0.01). Significantly, it also reduced TI stimulated by hGH at 10 ng/ml (plus 0.5% HPS) by 38±6% (P < 0.05). A monoclonal Ab to heart worm, prepared by a similar method, did not reduce TI stimulated by either IGF-I or hGH (Fig. 4). The monoclonal Ab to IGF-I had no effect when added alone (Table I). Insulin (plus 0.5% HPS) at 1,000 ng/ml (10-fold the IGF-I dose) increased TI by 528±57%. Neither the specific IGF-I monoclonal nor the unrelated heart worm Ab had any effect on insulin-stimulated DNA synthesis (Table I). The fibroblast mitogen EGF was
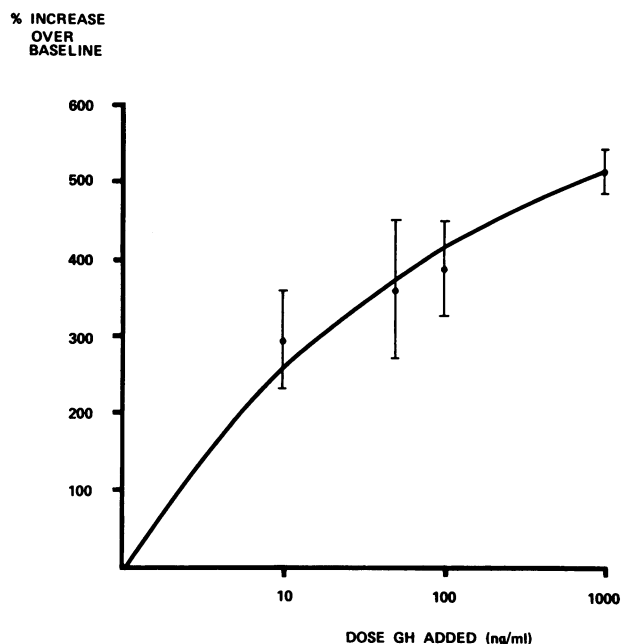
% INCREASE OVER BASELINE

Figure 2. hGH dose response in the presence of 0.5% HPS. The results are expressed as percentage increase of [³H]TI over baseline counts for SFM (±SEM) (n = 4). Statistical significance of the dose-response effect was determined using Friedman two-way analysis of variance (P < 0.01).

added at 10 ng/ml in the presence of 0.5% hypox serum. An increase in TI of 3,050±50% was seen (n = 2), with no blunting in the presence of the monoclonal Ab to IGF-I (1:1,000).

The effect on TI of increasing concentrations of the monoclonal Ab to IGF-I was examined in the presence of hGH or IGF-I (plus 0.5% hypox serum), both at 10 ng/ml (Fig. 5). TI induced by IGF-I and hGH was reduced by the Ab in a dose-dependent and parallel fashion at concentrations of the Ab of 1:5,000, 1:1,000, and 1:500 (P < 0.01 for each).

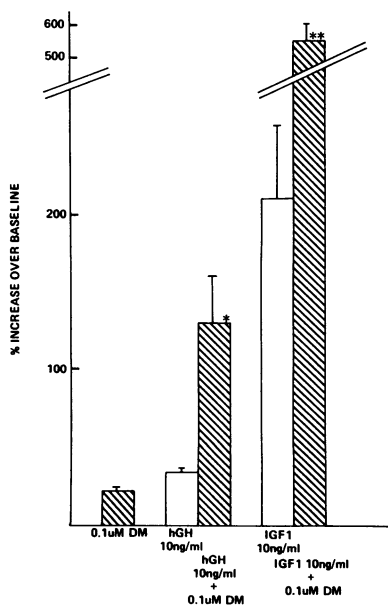To characterize the likely chronological relationship between growth hormone addition and resultant IGF-I–induced

Figure 3. Addition of 0.1 μM DM to IGF-I 10 ng/ml and hGH 10 ng/ml in the presence of 0.5% HPS. The results are expressed as percentage increase of [³H]TI over baseline counts for SFM (±SEM) (n = 3). *Compared with hGH 10 ng/ml alone P < 0.01. **Compared with IGF-I 10 ng/ml alone P < 0.01.
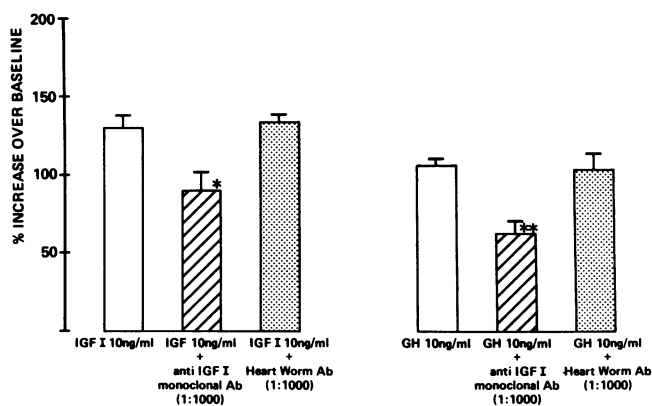

Figure 4. The effect of the monoclonal Ab to IGF-I (1:1,000) on [³H]-TI stimulated by IGF-I 10 ng/ml or hGH 10 ng/ml in the presence of 0.5% HPS. The results are expressed as percentage increase over baseline counts for SFM (n = 8) (±SEM). Compared with IGF-I 10 ng/ml alone *P < 0.01. and with hGH 10 ng/ml alone **P < 0.05.

DNA synthesis, we compared the time course of TI stimulated either by IGF-I or hGH at 10 ng/ml (in the presence of 0.5% hypox serum) when added to the fibroblasts at regular intervals over a 28-h time course. Thymidine was added for the last 3 h as before. Fig. 6 shows the results of a representative of the three experiments performed in triplicate. The effect of hGH lagged behind that of IGF-I by 4–10 h, reaching a similar peak of 1,800 cpm at 28 h, compared with 22 h for IGF-I.

Cell replication experiments

As a specific index of mitogenesis we examined cell replication stimulated by hGH and IGF-I in four experiments as shown in Fig. 7. 0.5% hypox serum alone, IGF alone, or hGH alone had no effect on cell replication compared with SFM (data not shown). However, in the presence of 0.5% HPS, IGF-I at 10 ng/ml and hGH at 10 ng/ml gave a similar stimulation of cell replication with a mean increase over baseline cell counts for

Table I. Effect of Insulin and IGF-I Monoclonal Ab, Alone or in Combination

	TI fibroblasts (percent increase over baseline for SFM)		Cell replication into (percent increase over baseline for SFM)	
	Mean (n = 3)	SE	Mean (n = 3)	SE
Monoclonal Ab to IGF-I	2	2	17	17
Heart Worm Ab	0.1	8	14	10
Insulin (1,000 ng/ml)	528	57	201	30
Insulin (1,000 ng/ml) and monoclonal Ab to IGF-I	634	49	210	14
Insulin (1,000 ng/ml) and Heart Worm Ab	532	28	206	16

Mitogenesis was determined in cultured skin fibroblasts as increase in DNA synthesis (expressed as percent increase in [³H]TI) and by cell replication (expressed as percent increase in cell number). Unrelated heart worm Ab was used as control. All additives were in the presence of 0.5% hypox serum.
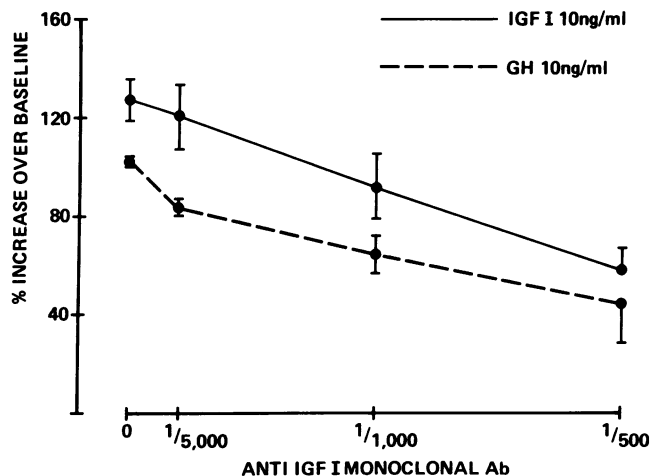
Figure 5. The effect of increasing concentrations of the monoclonal Ab to IGF-I on TI stimulated by IGF-I 10 ng/ml and hGH 10 ng/ml. The results are expressed as percentage increase of [^{3}H]TI over baseline counts for SFM (n = 4) ($\pm$SEM). Statistical significance of the effect of the Ab on TI stimulated by both IGF-I and hGH was determined using Friedman two-way analysis of variance (P < 0.01 for each).

SFM of 169±22% and 172±12%, respectively. Addition of the monoclonal Ab to IGF-I reduced cell counts stimulated, respectively, by IGF-I and hGH to 42±8% and 32±5% of these values (Fig. 7) (P < 0.01 for each). The addition of unrelated

heart worm Ab to IGF-I and hGH had no effect on the stimulated cell counts. Neither heart worm Ab nor the monoclonal Ab to IGF-I had any effect on cell replication when added alone. Nor did they have any effect on cell replication stimulated by insulin at 1,000 ng/ml (Table I).

Discussion

We have shown that hGH stimulates mitogenesis in human fibroblasts in the absence of exogeneous IGF-I, as shown by TI and cell replication studies. The action of hGH on DNA synthesis required competence factors present in HPS as has been previously described for IGF-I's mitogenic action (10, 19, 22). Platelet-derived growth factor (PDGF) is such a competence factor recognized as being necessary for stimulation of human fibroblast growth by several hormones including IGF-I (11, 19). Furthermore, PDGF, fibroblast growth factor, and macrophage growth factor can stimulate the production of somatomedin by cultured human fibroblasts (11). Consequently, the effects of PDGF on DNA synthesis in this system are blunted by IGF-I monoclonal Ab (23). It is thus possible that PDGF and other unidentified factors in the HPS were able to stimulate small quantities of local IGF-I secretion. This cannot be excluded, since our assay did not measure IGF-I in conditioned media. But, consequent effects on mitogenesis were minimal, as seen in Fig. 1, suggesting that the primary role of HPS in our system was the provision of competence factors, allowing synergism with hGH or IGF-I. Culture density, cell passage number, the time since the last media change, and the
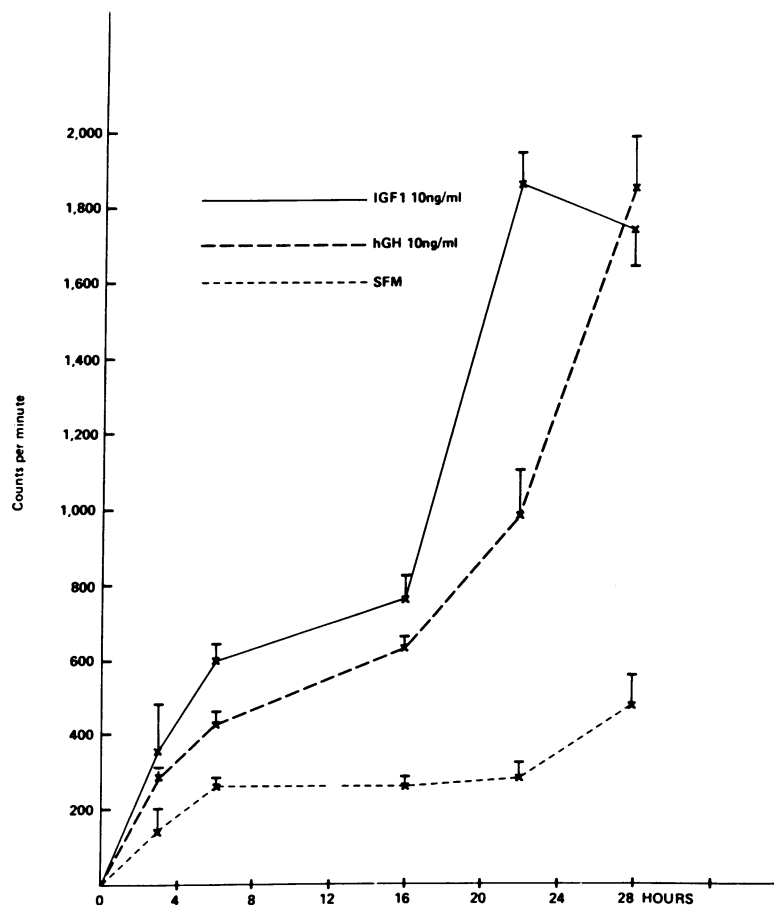

Figure 6. [^{3}H]TI stimulated by IGF-I 10 ng/ml and hGH 10 ng/ml added at intervals over 28 h, and labeled thymidine added for last 3 h in each case. The results show a typical representative of the three experiments with standard deviation bars shown for triplicates.
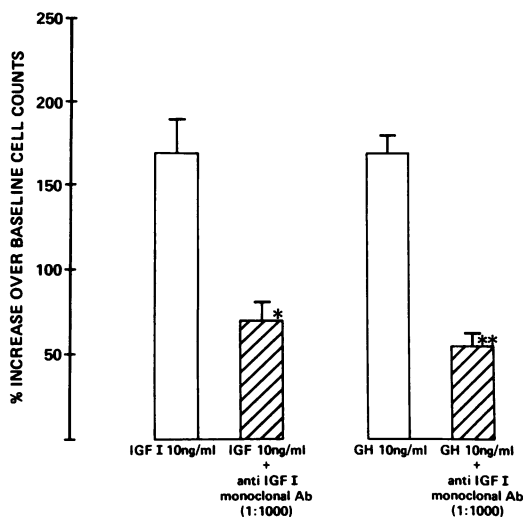
Figure 7. Cell replication stimulated by IGF-I 10 ng/ml and hGH 10 ng/ml and the effect of addition of the monoclonal Ab to IGF-I. The results are expressed as percentage increase over baseline cell counts for SFM (±SEM) ($n = 4$). *Compared with IGF-I 10 ng/ml alone $P < 0.01$. **Compared with hGH 10 ng/ml alone $P < 0.01$.

duration of incubation are important variables controlling IGF-I production by cultured human fibroblasts (12), and they were controlled as much as possible in our experiments. Nevertheless, significant quantitative variation in the GH effect was seen between sets of experiments, each of which was performed on a single cell line. As described previously for other biological effects, including insulin binding to human fibroblasts (24), it is likely that fibroblast cell lines differ in their GH responsiveness. This may represent variations in GH receptor status, or a postreceptor-regulated phenomenon.

The effect of hGH was dose dependent, with half-maximal effect at 10 ng/ml or 0.5 nM, in the physiologic range and consistent with the affinity for the fibroblast hGH receptor (13). In previous studies (22), IGF-I has demonstrated a half-maximal effect on DNA synthesis in human fibroblasts at a similar concentration (10 ng/ml or 1.3 nM). We therefore compared hGH with IGF-I at 10 ng/ml for each. In each of the studies described, hGH mimics the IGF-I effect at these doses, suggesting similar mechanisms, and further, suggesting molar equivalence. Whereas TI is widely accepted as an index of DNA synthesis, thymidine transport and thymidine kinase activity can be sensitive to the growth state of the cells, so that measuring TI into DNA may not always accurately reflect DNA synthesis (25). We therefore examined cell replication as a specific index of mitogenesis to confirm our findings. To further study whether hGH was exerting its mitogenic action on human fibroblasts via local IGF-I production we made use of the monoclonal Ab to IGF-I. The addition of this Ab blunted TI and cell replication stimulated by both hGH and IGF-I to a similar degree. The lack of any blunting effect by the monoclonal heart worm Ab supported the specificity of the monoclonal Ab to IGF-I. Furthermore, whereas both hGH- and IGF-I–stimulated DNA synthesis were blunted by the monoclonal Ab to IGF-I, neither insulin nor EGF-stimulated DNA synthesis were affected by the Ab. Insulin has previously been shown to mimic mitogenic effects of IGF-I at high concentrations (26). It is not clear from these studies whether

insulin acted at its own or the IGF-I receptor, though recent studies using monoclonal receptor Ab (24) suggest that it probably acts via its own receptor at low doses (< 10 ng/ml) and via the type I (IGF-I) receptor at higher doses in this system. We used a high dose to promote action via the type I receptor. The fact that its effects were not blunted by the monoclonal Ab to IGF-I provide further evidence for specificity of the IGF-I Ab effects. This is further supported by the failure of the Ab to inhibit DNA synthesis stimulated by EGF, known to act via its own specific receptor (27). Though the monoclonal Ab to IGF-I has ~ 30% cross-reactivity with IGF-II, this peptide is not a known product of human fibroblasts, and in any case is only weakly mitogenic (21). It is therefore unlikely to be a significant contributor to mediation of the hGH mitogenic effect.

Using different concentrations of the monoclonal Ab to IGF-I, DNA synthesis stimulated by hGH and IGF-I was inhibited in a dose-dependent and parallel fashion by the Ab. This provides further evidence that GH and IGF-I are acting via similar mechanisms. If hGH simply required the presence of small quantities of IGF-I to permit an independent direct effect, then parallel inhibition by the IGF-I monoclonal Ab would not be expected. Nevertheless, since the monoclonal Ab to IGF-I failed to be totally inhibitory, the possibility exists that GH's mitogenic effects are in part direct, and synergistically dependent on low concentrations of locally produced IGF-I, possibly under GH control. Such a concept has recently been proposed in 3T3 fibroblast differentiation to adipocytes (28), and in chondrocyte differentiation (29). There are other possible explanations for the failure of the monoclonal Ab to IGF-I to totally inhibit hGH mitogenesis: secreted IGF-I may have significant autocrine action and remain at least in part within the cells, so that it is not reached by the Ab. The use of specific antireceptor Abs may resolve this issue. The recent finding of IGF binding protein secretion by cultured human fibroblasts (30, 31) raises the possibility that some of the secreted IGF-I was not available to the added Ab, as it was bound to binding protein, from which it was released to the receptor. Also other growth factors present in the HPS may synergistically stimulate fibroblast replication in the presence of small quantities of IGF-I.

The synergistic action of DM and IGF-I in stimulating mitogenesis in cultured human fibroblasts has been previously described (22), and we have shown a similar threefold increase in TI stimulated by hGH with the addition of 0.1 μM DM. This further similarity in hGH- and IGF-induced DNA synthesis in these cells supports the likelihood of hGH acting via local IGF production, though these experiments do not exclude the possibility of synergism between hGH and DM before IGF-I synthesis.

The time course over 28 h for TI stimulated by IGF-I and hGH showed a time lag for the GH-stimulated effects of 4–10 h. It is of interest to compare this in vitro time lag with the in vivo delay of 12 h that D'Ercole and co-workers noted between injecting GH and observing the maximal rise of IGF-I extractable from tissues in hypophysectomised rats (32). The time lag noted in our studies, therefore, further supports the notion that the mitogenic effect of hGH in human fibroblasts is entirely mediated by IGF-I synthesized locally during the 4–10-h period. Further studies with cDNA probes for IGF-I will allow determination of the components of the time lag from hGH exposure to DNA transcription and protein synthesis. We were

unable to measure IGF-I in the conditioned media aspirated at the time of the cell harvesting. (The lower sensitivity limit of the Immunonuclear Corp. kit used was 5 ng/ml). IGF-I has been measured frequently in the media of cultured human fibroblasts stimulated by a variety of hormones (9, 11, 12). However, it was not detected in the media of cultured bovine chondrocytes where GH appeared to be stimulating growth via local IGF-I production by the chondrocytes (33). It is possible that in some instances the locally produced IGF-I binds immediately to receptor sites so that the media concentration remains low, or that locally produced binding proteins (30, 31) interfere with measurement. However, our monoclonal Ab studies clearly indicate local production of IGF-I, to which the Ab binds. A recent study has characterized the somatomedin–IGF-I–like peptide secreted from human fibroblasts, showing it to be of higher molecular weight and biologically more potent than circulating IGF-I (34). However, it is functionally similar to IGF-I and binds to the IGF-I monoclonal Ab used in our studies (34). Though we have not directly demonstrated secretion of a biologically active IGF under the influence of hGH, blunting of the GH-induced effects by this Ab indicates that GH-induced mitogenesis is at least in part mediated by local production of such a biologically active IGF-I–like peptide.

These studies of the relationship between hGH and its mitogenic response in human tissues further support a direct link to local tissue secretion and availability to IGF-I as the mediator of hGH action, or at least provide a necessary local component for maximal GH-induced mitogenesis. We have (i) provided further evidence for mitogenic effects of hGH in cultured skin fibroblasts, mimicking effects of IGF-I at similar half-maximal doses; (ii) demonstrated that hGH's mitogenic action on cultured human fibroblasts is mediated, at least in part, via local IGF-I production, as indicated by (a) blunting of hGH effects by a monoclonal Ab to IGF-I and (b) demonstration of a consistent time lag between hGH-induced and IGF-I–induced mitogenesis. Our findings are consistent with a GH-dependent paracrine–autocrine role for IGF-I, and the cell surface and intracellular events that occur require further investigation.

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