

Human growth hormone stimulates proliferation of human retinal microvascular endothelial cells *in vitro*

(somatotropin/diabetic retinopathy/neovascularization)

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ABSTRACT Growth hormone (GH) has been implicated in the pathogenesis of proliferative diabetic retinopathy. We sought to determine whether this could be mediated by an effect of GH on proliferation of endothelial cells, and, for this purpose, established long-term cultures of human retinal microvascular endothelial cells (hREC) from normal postmortem human eyes. High-purity (>95%) hREC preparations were selected for experiments, based on immunofluorescence with acetylated low density lipoprotein (LDL) and anti-factor VIII-related antigen. Growth requirements for these cells were complex, including serum for maintenance at slow growth rates and additional mitogens for more rapid proliferation. Exposure of hREC to physiologic doses of human GH (hGH) resulted in 100% greater cell number vs. control ($P < 0.01$) but could be elicited only in the presence of serum. When differing serum conditions were compared, hGH stimulated [³H]thymidine incorporation up to 1.6- to 2.2-fold under each condition and increased DNA content significantly in the presence of human, horse, and fetal calf serum. Depending on the culture conditions used, the threshold hGH concentration for significant stimulation of hREC proliferation was 0.4–4 $\mu\text{g/liter}$. In contrast, proliferation of human umbilical vein endothelial cells was not significantly altered by hGH added to concentrations as high as 200 $\mu\text{g/liter}$. In summary, hREC respond to physiologic concentrations of hGH *in vitro* with enhanced proliferation. This specific effect of GH on retinal microvascular endothelial cells supports the hypothesis of a role for GH in endothelial cell biology.

Mechanisms underlying the development of proliferative diabetic retinopathy (PDR) remain poorly understood (1–3). The observation that pituitary ablation may reverse or slow the progression of PDR, first made >35 years ago, has raised the question of a role for a pituitary-derived or -dependent factor in this disease process (4–8). As a consequence of this and of abnormalities in both the quantity and pattern of secretion of growth hormone (GH) in poorly controlled diabetes (9–12), GH has been suggested as a possible mediator of the effect of pituitary ablation on PDR, potentially via insulin-like growth factors (IGFs) (2, 13). However, there is increasing awareness that GH acts not only via systemic IGFs but also directly without systemic mediation at a greater number of target tissues than has been recognized (14, 15). Therefore, we sought to determine whether GH has effects directly on microvascular endothelial cells (EC) derived from the retina. Use of cells differing in either species or tissue of origin is a potentially confounding variable (16–23), while studies on human microvascular EC have been limited (24–27). We now report the establishment of long-term cultures of such cells from the postmortem human retina (hREC) and demonstrate that these cells respond to physiologic concen-

trations of biosynthetic human growth hormone (hGH) with enhanced proliferation.

MATERIALS AND METHODS

Reagents were obtained from the following sources: fetal calf serum (FCS), horse serum (HS), trypsin, medium 199 (M199) with 25 mM Hepes buffer, and bovine serum albumin from GIBCO; EC growth supplement, human fibronectin, collagenase type II-S from Sigma; acetylated low-density lipoprotein (LDL) labeled with 1,1'-dioctadecyl-1-(3,3,3',3'-tetramethyl)indocarbocyanine perchlorate (DiI-acetylated LDL) from Biomedical Technologies (Stoughton, MA); Mito+ serum extender from Collaborative Research; rabbit antiserum to human factor VIII from Calbiochem. Biosynthetic hGH was provided by Genentech, San Francisco; pituitary corticotropin (ACTH), by the National Hormone and Pituitary Program; and biosynthetic IGF-1 (Amgen Biologicals), by S. Chernauek. Human serum (HuS) was obtained from healthy male volunteers, ages 21–40. The endogenous concentration of hGH in the human sera used was determined by hGH RIA and reported with each individual experiment, and GH-like immunoreactivity in FCS was determined by using an ovine GH RIA (28).

hREC Isolation and Culture. Postmortem human eyes were obtained from the Cincinnati Eye Bank for Sight Restoration and from the National Disease Research Interchange (Philadelphia). The donors were previously healthy victims of trauma, ages 2–37 years of both sexes. Exclusion criteria included sepsis, malignancy, and systemic diseases. Cells were isolated and established in culture by techniques modified from previously described methods (19, 20, 24, 26, 29). Briefly, an incision was made at the ora serrata under sterile conditions, and the vitreous was removed. The neurovascular retina was separated from the optic nerve, transferred to a tube containing chilled M199 with 25 mM Hepes and briskly stirred in five changes of medium. The retinas then were minced, vigorously passed by syringe through an 18-gauge needle, and the capillaries were separated by sieving through nylon mesh of sequentially smaller pore size (200- to 50- μm pores). Retinal capillaries retained by the last nylon screen were partially digested with collagenase (10 $\mu\text{g/liter}$ in M199 containing 1% bovine serum albumin) for 0.5–1.5 hr. Small fragments of capillaries were suspended in growth medium (M199 containing 20% HS, EC growth supplement at 20 mg/liter, heparin at 90 mg/liter, 2 mM L-glutamine, and

Abbreviations: DiI-acetylated LDL, acetylated LDL labeled with 1,1'-dioctadecyl-1-(3,3,3',3'-tetramethyl)indocarbocyanine perchlorate; FCS, fetal calf serum; GH, growth hormone; hGH, human GH; EC, endothelial cell(s); hREC, human retinal microvascular endothelial cell(s); hUVEC, human umbilical vein endothelial cell(s); HS, horse serum; hGH, human growth hormone; HuS, human serum; IGF, insulin-like growth factor; LDL, low density lipoprotein; M199, medium 199; PDR, proliferative diabetic retinopathy; ACTH, corticotropin.

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mitogen serum extender at 1 ml/liter). The cell suspension was then plated in dishes coated with 0.5% gelatin and fibronectin ($1 \mu\text{g}/\text{cm}^2$). This medium was used for all procedures except as otherwise noted.

Cellular outgrowth from small microvascular fragments began on days 3–4 and consisted of colonies of both EC and pericytes and, commonly, a mixture of both cell types. To obtain more homogeneous preparations, we initially used techniques previously applied to retinal EC isolation, including (i) "weeding" of unwanted colonies (30) and (ii) Percoll density gradient centrifugation (22, 26). However, to obtain a sufficient yield of hREC, it was necessary to use fluorescence-activated cell sorting (Becton Dickinson model 440) after labeling cells with DiI-acetylated LDL (at $10 \mu\text{g}/\text{liter}$ of culture medium for 12 hr; ref. 31). Cells were maintained in growth medium until confluent. For subculture, cells were trypsinized (0.25%) and then split 1:3–4. The purity of each culture was monitored frequently by morphology based on light microscopy and by fluorescent staining with DiI-acetylated LDL and anti-factor VIII-related antigen. EC from human umbilical vein (hUVEC) were isolated by established techniques (32) and used as a positive control for both staining procedures.

Measurement of Cell Number and Proliferation. All experiments were performed with cells that were between passages 4 and 7 at an initial density of $1.3\text{--}1.8 \times 10^4$ cells per cm^2 . Plating medium was M199 containing 2 mM L-glutamine and 20% HS or FCS. The day after plating, the medium and unattached cells were removed, the cells were washed once with M199, and incubation of the cells was initiated with medium containing serum and hormones for 4–9 days as described below. Hormone concentrations shown in the figures represent the concentration added (in $\mu\text{g}/\text{liter}$; to convert to pM, multiply by 50) and are not corrected for hormone contributions from added serum. Cell proliferation was measured by: (i) cell counting (Nebauer hemocytometer); (ii) determination of cellular DNA content using a modified microfluorometric method with Hoechst fluorochrome 33258 (33, 34); and (iii) determination of [^3H]thymidine (80 Ci/mmol; $0.2 \mu\text{Ci}/\text{ml}$ of medium; $1 \text{ Ci} = 37 \text{ GBq}$) incorporation into DNA after a 12-hr incubation (35).

Data Analysis. Data are expressed as means \pm SEM. Data were analyzed by two-way analysis of variance (ANOVA) with Duncan's new modified range test (see Fig. 3) or by one-way ANOVA (Figs. 4–6) with Dunnett's modified *t* method for comparison of multiple groups to a single control or by unpaired *t* test (effect of glucose on hREC proliferation).

RESULTS

hREC Isolation and Culture. In preliminary experiments to establish a cell isolation procedure and culture conditions based on methods reported for culture of EC from both human and nonhuman microvessels, we observed a low rate of successful culture (<10% of attempted isolations) as assessed by serial propagation of high-purity EC cultures. Similarly to bovine retinal pericytes (36), human retinal pericytes strongly inhibited proliferation of hREC in our system. In mixed culture, acetylated LDL-staining cells ceased to proliferate at 40–50% of confluence, whereas in pure EC culture, proliferation continued until confluence had been achieved. Purification using cell sorting based on labeling of EC with the fluorescent probe DiI-acetylated LDL provided a higher purity of viable cells than did the alternatives and was used in subsequent isolations. In four sortings, the yield of DiI-acetylated LDL-stained cells ranged between 9% and 20% of the total sorted. This method allowed long-term culture of high-purity preparations of hREC (>95% cells stained positive for acetylated LDL and factor VIII) from

four donors; two of these preparations have been maintained thus far for 11 passages. Even at that stage, hREC grew in a monolayer with cobblestone-like morphology and continued to exhibit cytoplasmic perinuclear granular fluorescent staining with both DiI-acetylated LDL and anti-factor VIII-related antigen (Fig. 1). hUVEC stained with anti-factor VIII-related antigen served as a positive control, whereas human pericytes and fibroblasts failed to fluoresce (not shown).

hGH and hREC Proliferation: Serum Requirement for an Effect of hGH. Initially, the proliferative response of hREC to different concentrations of hGH was measured by cell counting after incubation in growth medium from which serum had been omitted, in comparison with growth medium modified to 2.5% HS. In the absence of serum, hGH did not exert a mitogenic effect on hREC (Fig. 2). However, in the presence of 2.5% HS, hGH at as low a concentration as $1.2 \mu\text{g}/\text{liter}$ (0.06 nM) resulted in increased cell number by almost 2-fold more than in the absence of GH during a 4-day incubation.

Comparison of the Effect of hGH on [^3H]Thymidine Incorporation and DNA Content. A significant mitogenic effect on

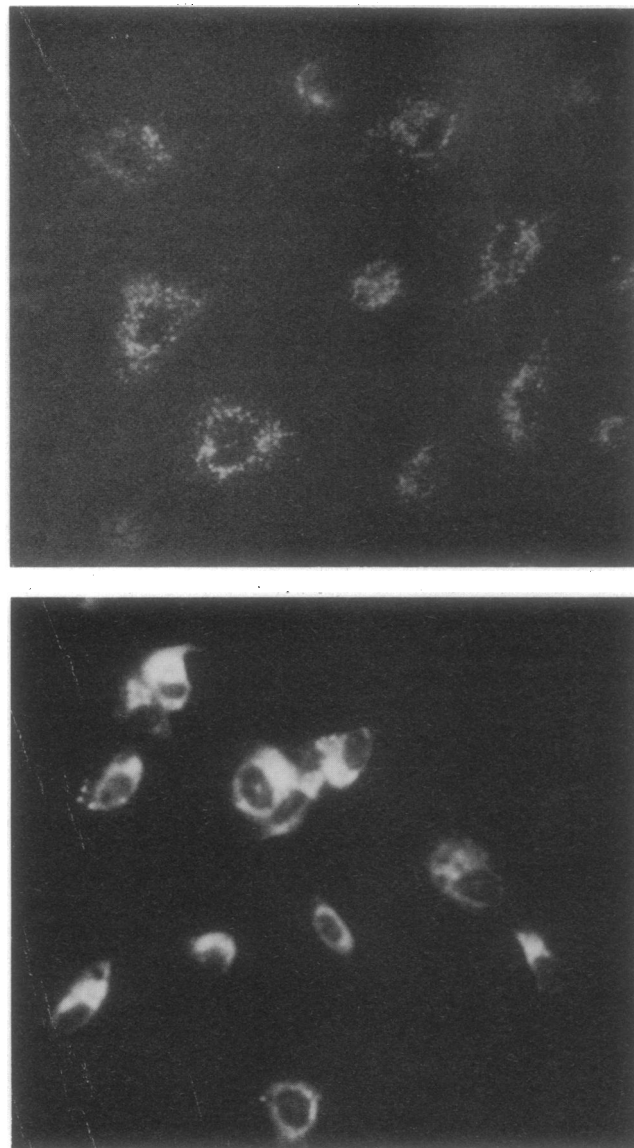


FIG. 1. Fluorescent staining of representative preparations of hREC (after passage 10). (Upper) hREC labeled with DiI-acetylated LDL. ($\times 800$.) (Lower) hREC labeled with rabbit anti-human VIII-related antigen and fluorescein coupled to goat anti-rabbit IgG (Calbiochem). ($\times 380$.)

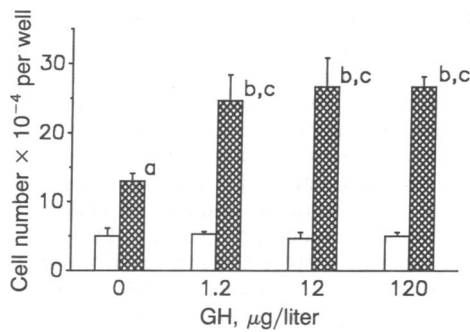


FIG. 2. Effect of serum on biosynthetic hGH stimulation of hREC proliferation. Cells in passage 7 from donor 1 were plated at a density of 1.8×10^4 per cm^2 (4×10^4 per well) and exposed to hGH for 4 days. At that time, cell number was then determined on all samples in triplicate. □, No serum added; ■, 2.5% HS added; a, $P < 0.05$ vs. control (no serum); b, $P < 0.01$ vs. control (no serum); c, $P < 0.01$ vs. absent GH (2.5% HS).

hREC was demonstrable (by both DNA content and [^3H]thymidine incorporation) at the lowest hGH concentration tested, 1.2 $\mu\text{g/liter}$ (0.06 nM), under each of several serum conditions tested (growth medium in which HuS or HS was substituted to either 2.5% or 10% concentration); data are shown only from incubations in 2.5% HS (Fig. 3). Since the concentration of hGH in the HuS used was 0.2 $\mu\text{g/liter}$, the endogenous hGH contribution to the final hGH concentration when serum was added to a final concentration of 2.5% or 10% was negligible (0.005 and 0.02 $\mu\text{g/liter}$, respectively).

Threshold for hGH Stimulation of hREC Proliferation. Because the proliferative response appeared to be very sensitive to hGH, dose-response studies on the proliferation of hREC were subsequently conducted extending the hGH concentration range downward (0.1–200 $\mu\text{g/liter}$; Fig. 4) under conditions specifically selected to provide slower baseline proliferation. This was achieved by using M199 containing either 20% FCS or 15% pooled HuS and 2 mM L-glutamine with omission of the other growth supplements (heparin, EC growth supplement, and mitogen serum extender). Serum conditions were selected for these experiments as those necessary to maintain cell attachment in the absence of

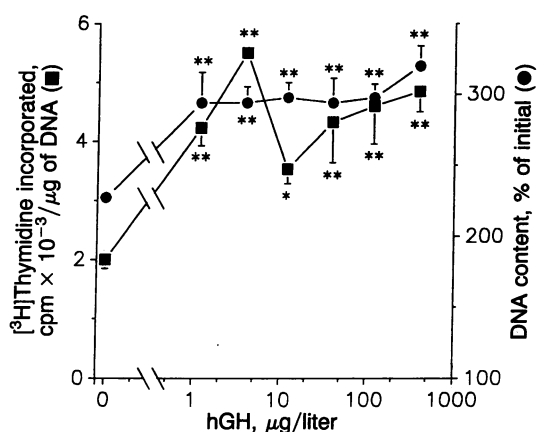


FIG. 3. Comparison of the effect of biosynthetic hGH on hREC proliferation measured by both [^3H]thymidine incorporation and DNA content. Cells in passage 6 from donor 1 were plated at a density of 1.3×10^4 per cm^2 (corresponding to 270 ng of DNA per well) and incubated for 6 days in 2.5% HS. During the last 12 hr, medium containing [^3H]thymidine (0.2 $\mu\text{Ci/ml}$) was added. Cells were then harvested to measure both [^3H]thymidine incorporation (left ordinate) and DNA content (right ordinate). All samples were analyzed in triplicate except for duplicate determinations of [^3H]thymidine incorporation at 400 μg of GH per liter. *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control (analysis of variance).

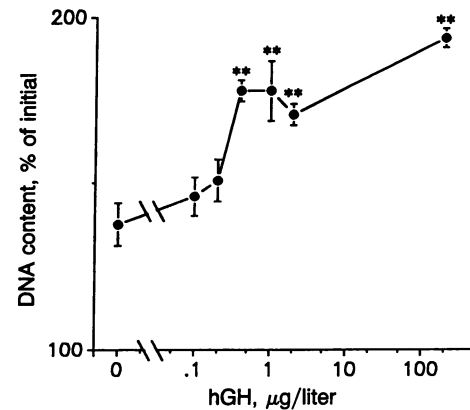


FIG. 4. Effect of low concentrations of biosynthetic hGH on hREC proliferation. Cells in passage 7 from donor 2 were plated at a density of $1.5 \times 10^4/\text{cm}^2$ (corresponding to 310 ng of DNA per well) and incubated in the presence of 20% FCS for 4 days with omission of other growth factors (see text), at which time they were harvested and the DNA content was determined (all samples in quadruplicate). *, $P < 0.01$ vs. control.

the supplements. GH-like immunoreactivity measured 64 $\mu\text{g/liter}$ in the FCS (providing an endogenous bovine GH concentration of 13 $\mu\text{g/liter}$ from 20% FCS), while that in the pooled HuS measured 0.5 $\mu\text{g/liter}$ (adding 0.075 $\mu\text{g/liter}$ to the hGH concentration in the culture well). In the presence of 20% FCS, the mean DNA content per well increased even at the lowest added hGH concentration tested, with the effect becoming statistically significant at 0.4 $\mu\text{g/liter}$. Indeed, the effect at 0.4 $\mu\text{g/liter}$ of hGH was indistinguishable from that at all higher concentrations up to 200 $\mu\text{g/liter}$. As an indication of the specificity of the effect of hGH, the effect of ACTH at a supraphysiologic concentration was tested during the same experiment; 1 μM ACTH did not alter hREC proliferation (410 ± 10 ng of DNA per well in ACTH-treated cells vs. 430 ± 20 in controls). When 15% HuS was substituted under the same otherwise deprived conditions, mean DNA content per well increased in hREC cultures beginning at 1 μg of hGH per liter, with the effect becoming significant at 4 $\mu\text{g/liter}$ (Fig. 5). There was only minimal further effect upon addition of up to 200 $\mu\text{g/liter}$ of hGH. A similar effect on proliferation was observed when IGF-1 was added at 200 $\mu\text{g/liter}$. In contrast, hUVEC that had been maintained in culture for a

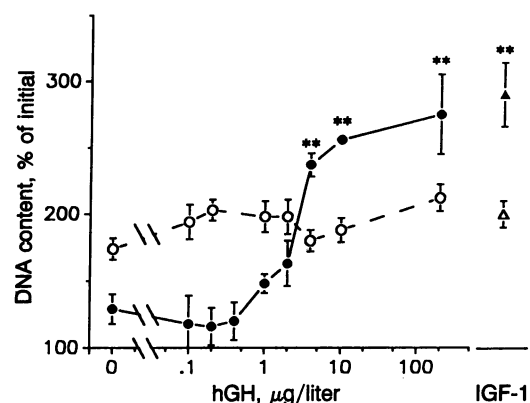


FIG. 5. Comparison of hGH and IGF-1 effects on hREC and hUVEC proliferation. hREC (●) in passage 7 from donor 2 were plated at a density of 1.1×10^4 per cm^2 (corresponding to 220 ng of DNA per well), and hUVEC (○) in passage 7 were plated at a density of $1.9 \times 10^4/\text{cm}^2$ (390 ng of DNA per well); both were incubated in the presence of 15% pooled HuS for 9 days with omission of other growth factors (see text). Cells were harvested, and DNA content was determined in quadruplicate. *, $P < 0.01$ vs. control.

similar number of passages and grown under the same conditions failed to show a significant response to either hGH or IGF-1.

Glucose and hREC Proliferation. To assess the potential for synergy between hGH and glucose in the diabetic milieu, the effect of 25 mM glucose was compared with that of 5 mM glucose on hREC DNA content after a 9-day incubation (data pooled from two experiments in which cells were plated at a density of 3000 per cm²; $n = 10$). At 5 mM glucose, the final DNA content per well was 3.3 ± 0.2 times the initial DNA content, whereas at 25 mM glucose, the final DNA content was 1.8 ± 0.2 times the initial content ($P < 0.002$). Because glucose and hGH had opposite rather than similar effects, potential interactions have not been pursued further.

DISCUSSION

There are at least two lines of evidence to support a role for a pituitary-derived or pituitary-dependent factor in the development of PDR and a third line of evidence to specifically implicate hGH: (i) the effect of pituitary ablation on PDR (4–6), (ii) the relationship of development of retinopathy to puberty or adolescence or both, and (iii) findings in ateliotic dwarfs. The effect of pituitary ablation on PDR is controversial but is supported by two controlled studies and a recent long-term follow-up (7, 8, 37). The latter demonstrated far greater reversal of proliferative changes and preservation of vision (unrelated to thyroxine, glucocorticoid, or sex steroid replacement) than one would expect in relation to the progressive course of untreated PDR, as documented, for example, in the control group of the Diabetic Retinopathy Study (38). Use of pituitary ablation has been documented in >900 patients with PDR (39). Given that the mediator of the effect and the degree of hormonal ablation necessary were not known and assays for pituitary peptides were less sensitive than those today, adequacy of ablation of specific putative mediator(s) and hence whether the data fairly addressed the role of a pituitary factor is difficult to judge. It should not be surprising that variable results were obtained. Subsequently, the prevalence of retinopathy has been shown in a 6- to 23-year-old population to be greater in those older than 15 years, when the duration of diabetes had been taken into account (40). This further supports a role in retinopathy for a factor present in both sexes that is related to either growth or sexual development, most likely a pituitary-derived or -dependent factor. Studies in ateliotic dwarfs demonstrated a high frequency of diabetes. Despite this, in comparison to matched control patients with type II diabetes, they had a markedly lower frequency of retinopathy and did not have the skeletal muscle capillary basement membranes thickening found in patients with diabetes (41).

There are numerous observations that hGH is elevated in poorly controlled diabetes mellitus, which focused attention on the role of the hGH axis as the mediator of pituitary ablation in PDR (9–12, 42). These have led to two lines of investigation, concerning the IGFs and alterations in neuropharmacologic control of hGH secretion. Plasma and vitreous IGF-1 concentrations have been reported higher in diabetic patients with PDR than in those without (43, 44), and IGF-1 has been shown to alter retinal endothelial cell chemotaxis and secretion of plasminogen activator (25, 26). There is controversy whether elevated concentrations of IGF-1 reflect increased production of or altered vascular permeability to IGF-1 (15, 43, 44). *In vivo* human pharmacologic studies have demonstrated increased responsiveness of pituitary hGH secretion to thyrotropin-releasing hormone (a hypothalamic peptide that ordinarily does not stimulate GH secretion) and to arginine in patients with retinopathy (45, 46). These studies provide evidence of altered control of hGH

secretion without addressing whether hGH has biologic effects that may modify the course of retinopathy.

It is in this context that the question arises whether pituitary hormones, specifically GH, have direct effects on the endothelium. The present studies demonstrate that cells from the human retinal endothelium are indeed capable of responding to hGH under the conditions described. Both morphologic and functional features of endothelium were preserved under the conditions of study. The present studies do not distinguish whether the increased proliferation rate resulted from hGH action alone or whether hGH induced the secretion of another mediator by endothelial cells (e.g., IGF-1 or another factor), which in turn induced mitotic activity. The finding reported here is consistent with those of other investigators in the last several years that certain actions of GH occur directly at a number of target tissues rather than as a consequence of systemic IGF-1 secretion (14, 15).

The effect of hGH on hREC proliferation requires the presence of serum. This requirement may explain the lack of a GH effect in results reported by King and co-workers (23) studying bovine retinal endothelial cells under serum-free conditions. While species specificity of the cells cannot be excluded, the presence of serum would seem the more likely explanation. The nature of the specific component of serum necessary for this GH effect is unclear at this time.

The effect of hGH was numerically small in some experiments—e.g., a 30% increase when DNA content was used as the measure of cell number (Fig. 3). However, this corresponded to a simultaneously measured [³H]thymidine incorporation that was 2.3 times the basal rate (Fig. 3), and in other experiments a 2-fold increase over 4–9 days in either cell number or DNA content was found. Since mitoses are infrequent in endothelium (estimated turnover time $\approx 10,000$ days; refs. 47 and 48) and the exposure to these concentrations of GH is prolonged, even a seemingly small-magnitude effect in a brief experiment may reflect a process that compounds many times *in vivo* and has substantial implications for disease progression.

hREC are sensitive in a dose-dependent manner to exceedingly low hGH concentrations *in vitro*, 0.4–4 $\mu\text{g/liter}$. While there was a high content of bovine GH immunoreactivity in the experiments conducted in FCS, the species specificity of GHs is such that the biological activity contributed in this human cell system would be expected to be much smaller. The fact that we were able to measure a statistically significant response in a dose-dependent manner at markedly lower hGH concentrations argues for a relatively small biological effect of bovine GH on these human cells. The dose-response was shifted to the left in FCS compared to that in HuS, suggesting the effect of another component of one or both sera used, either a stimulator in the FCS or a suppressor in the HuS. The failure of EC from a different vascular source (the umbilical vein) to respond to hGH both supports the specificity of the response and suggests that this commonly used EC experimental model will not be an appropriate substitute for studying these interactions in human disease. To our knowledge, effects of GH on other vascular preparations *in vitro* have only been shown previously with non-endothelial, nonhuman material—i.e., myomedial cell proliferation and synthetic function in rabbit aortic explants (49, 50).

The dose dependence observed is comparable to the most sensitive responses to GH we are aware of described *in vitro* (51, 52). This GH concentration range is commonly exceeded *in vivo* not only in disease states, diabetes and acromegaly, but also in healthy people. The lowest concentration of hGH at which a mitogenic effect is seen, 0.4 $\mu\text{g/liter}$, is intermediate between the levels we found at baseline in patients with poorly controlled diabetes and normal control subjects, using

a highly sensitive hGH RIA (ref. 11; unpublished data). The *in vitro* conditions selected may lack factor(s) present *in vivo* that restrain the ability to respond to hGH. Such factors may also represent functional differences in the retinal microvasculature between individuals with and without diabetes and are presently under investigation. If found, they may provide a basis for a role for GH in the development of retinopathy while explaining the absence of retinopathy in a disease in which increased circulating GH is the major abnormality, acromegaly.

In summary, the present studies demonstrate that cells from the human retinal endothelium can exhibit a proliferative response to physiologic concentrations of hGH. To our knowledge, this is the first evidence that isolated EC of any source can respond to GH. An effect of hGH directly on hREC supports the hypothesis that GH elimination mediates the amelioration of diabetic retinopathy by pituitary ablation and suggests a possible role for GH in endothelial cell biology.

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