Impaired Synergism between Somatomedin C/Insulin-Like Growth Factor I and Dexamethasone in the Growth of Fibroblasts from a Patient with Insulin Resistance

CHERYL A. CONOVER, RAYMOND L. HINTZ, AND RON G. ROSENFELD

Department of Pediatrics, Stanford University Medical Center, Stanford, California 94305

ABSTRACT. To explore a possible mechanism for the diminished growth potential in a patient with an unusual form of insulin resistance, somatomedin C/insulin-like growth factor I (SM-C/IGF-I) and insulin stimulation of [³H]thymidine incorporation and cell replication were compared in skin fibroblasts from the patient (DF) and normal controls. There appeared to be no generalized abnormality in cellular responsiveness to growth factors. In both DF and control cells, SM-C/IGF-I (50 ng/ml), insulin (100 ng/ml), and epidermal growth factor (5 ng/ml) stimulated [³H]thymidine incorporation 5-, 2-, and 6-fold, respectively. Low concentrations of human hypopituitary serum (0.25%) enhanced the effectiveness of SM-C/IGF-I and insulin to a similar extent in both DF and control cells. On the other hand, 10% calf serum stimulated [³H]thymidine incorporation 37-fold in control cells, while DF cells were only 50% as responsive. Preincubation of control cells with dexamethasone (10⁻⁷ M) caused a marked synergistic increase in SM-C/IGF-I stimulated [³H]thymidine incorporation (15- to 20-fold in serum-free medium; 50- to 80-fold in 0.25% human hypopituitary serum). In contrast, preexposure to dexamethasone did not augment SM-C/IGF-I stimulation of thymidine incorporation into DNA of DF cells. Furthermore, the stimulation of cell replication by SM-C/IGF-I and insulin was potentiated by dexamethasone in control but not DF cultures. These data suggest that impairment of the synergistic action of glucocorticoids with SM-C/IGF-I and insulin regulation of fibroblast growth may be involved in the pathology of this insulin-resistant growth disorder. (*Pediatr Res* 22: 188-191, 1987)

Abbreviations

SM-C/IGF-I, somatomedin C/insulin-like growth factor I
EGF, epidermal growth factor
HHS, human hypopituitary serum
SFM, serum-free medium
BSA, bovine serum albumin
IGF-II, insulin-like growth factor II
DMEM, Dulbecco's modified Eagle's medium

We have previously demonstrated that both insulin and SM-C/IGF-I, at physiological concentrations, are mitogenic for hu-

man fibroblasts in culture (1). At nanomolar concentrations, each peptide is capable of acting through its own receptor to stimulate DNA synthesis and cell multiplication. Although SM-C/IGF-I appears to be the more potent mitogen for human fibroblasts at these concentrations, at supraphysiological concentrations insulin can cross-react with the type I IGF receptor and achieve the same maximal effect as SM-C/IGF-I. Moreover, both insulin and SM-C/IGF-I are synergistic with glucocorticoids in the stimulation of DNA synthesis and replication of normal human fibroblasts, suggesting that the two peptides have a common postreceptor regulatory site in the pathway for mitogenesis.

A recent report (2) described patient DF, a 17-yr-old man, as having growth retardation associated with an unusual form of insulin resistance. This patient had no circulating antibodies directed against either insulin or the insulin receptor, and there was no clinical or laboratory evidence of increases in any hormonal insulin antagonists. Furthermore, despite high circulating insulin values, insulin binding and metabolic responsiveness, as measured by 2-deoxyglucose uptake into fibroblasts, were normal. SM-C/IGF-I levels were within the normal range.

To explore a possible postreceptor mechanism for this growth disorder, SM-C/IGF-I and insulin stimulation of [³H]thymidine incorporation and cell replication were compared in skin fibroblasts from DF and normal controls, under both serum-free and glucocorticoid potentiating conditions.

MATERIALS AND METHODS

Materials. Thymidine [methyl-³H] (6.7 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Dexamethasone and BSA were purchased from Sigma (St. Louis, MO), and AquaMix was from WestChem (San Diego, CA).

Pure synthetic SM-C/IGF-I was provided by Dr. C. H. Li [San Francisco, CA (3)]. Human insulin was furnished by Eli Lilly Company (Indianapolis, IN) and EGF was purchased from Collaborative Research Inc. (Lexington, MA). The somatomedin preparation designated SM in the text was the Cohn fraction IV-I acid-ethanol extract purified short of isoelectric focusing, as previously described (4). This preparation is insulin-free and by specific radioimmunoassays (5, 6) the SM-C/IGF-I content was determined to be 25 µg/mg weight and the IGF-II content was 10 µg/mg weight. Earlier studies have shown that this SM has equivalent biological potency in the human fibroblast system to highly purified SM-C/IGF-I preparations (1, 7-10).

HHS was obtained from two donors with growth hormone deficiency documented by failure to raise serum growth hormone levels above 7 ng/ml following arginine and insulin stimulation. IGF-I levels in these sera were less than 25 ng/ml as determined by radioimmunoassay [normal adult levels range from 150-250 ng/ml (11)].

Received December 4, 1986; accepted March 3, 1987.

Correspondence Cheryl A. Conover, S-322 Department of Pediatrics, Stanford University Medical Center, Stanford, CA 94305.

Supported in part by NIH Grants AG01213, AM33190, AM24085, and AM28229 and the Juvenile Diabetes Foundation Grant 185199. R.G.R. is the recipient of a Research Career Development Award AM01275 from the NIH.

Cell cultures. Skin fibroblasts from a 17-year-old male patient, designated DF, were obtained from Dr. H. Dean [University of Manitoba, Canada (2)]. Apparently normal human skin fibroblasts from a 24-yr-old man (GM3652) and a 7-yr-old boy (N1493) were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ) and from the American Type Culture Collection (Rockville, MD), respectively. These control cell lines have been characterized extensively in our laboratory in metabolic and mitogenic studies (1, 7–10, 12).

Cells were cultured in DMEM, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 4 mM glutamine (Gibco, Grand Island, NY), and containing 10% supplemented calf serum (Hyclone Laboratories, Logan, UT) as described previously (7). Cells were trypsinized and plated in 35-mm plastic dishes (Corning, NY) for [³H]thymidine incorporation experiments and in 16-mm multiwells (Costar, Cambridge, MA) for replication experiments. Control fibroblasts were used between passages 8 and 14 [less than 30% of *in vitro* life span completed (10)]. DF fibroblasts were used between passages 5 and 10. Although the life span of DF fibroblasts was not investigated, there were no morphological signs of *in vitro* senescence at these passage levels.

[³H]thymidine incorporation. Four days following the last feeding, confluent fibroblast cultures were incubated in DMEM with 0.1% BSA and dexamethasone (10^{-7} M) or ethanol vehicle (0.2%) for 48 h. After this preincubation, the medium was changed to the SFM plus experimental additions. At 21 h, [³H]thymidine was added to give a final concentration of 0.2 µCi/ml, and the cells harvested as described in an earlier publication (7). In this system, [³H]thymidine incorporation has been shown to peak 21–23 h following stimulation of serum-starved human fibroblasts with SM-C/IGF-I or serum, independent of donor or culture age (7, 8, 10, 12). Results are expressed as the percentage of total counts in the incubation medium which are incorporated into acid-precipitable material per 10^6 cells.

Cell replication. Cells were plated sparsely in a 1:1 mixture (by volume) of Waymouth's medium (Gibco) and DMEM containing 2% calf serum. The following day, designated day 0, the cells were washed, cell number was measured, and the medium was changed to Waym:DMEM with 0.1% BSA plus experimental additions. On day 3, cell number was determined in duplicate wells with a Coulter Counter.

Statistics. Differences between two groups were analyzed using the Student's *t* test for two independent samples. Results were considered statistically significant when $p < 0.05$.

RESULTS

Effect of somatomedin, insulin, EGF, and serum on [³H]thymidine incorporation. The stimulation of [³H]thymidine incorporation in DF and control fibroblasts by SM, insulin, and EGF alone, or by SM and insulin in combination with low concentrations of serum is shown in Figure 1. In SFM, the addition of SM (50 ng/ml SM-C/IGF-I), insulin (100 ng/ml), and EGF (5 ng/ml) stimulated [³H]thymidine incorporation 5-, 2-, and 6-fold, respectively. There was no significant difference between DF and control fibroblasts in their responsiveness to these growth factors.

HHS, at very low concentrations, has been shown to enhance the mitogenic effect of SM-C/IGF-I and insulin in normal human fibroblasts (1). HHS (0.25%) alone stimulated [³H]thymidine incorporation 3-fold in control and DF cells. HHS potentiated SM action to 26-fold in control cells and to 16-fold in DF cells. Although the difference in responsiveness of DF and control cells to SM + HHS is significant in this experiment, this was not a consistent finding (see Fig. 2) and the magnitude of the effect in DF cells is within the normal range for cultured human fibroblasts (8). HHS enhanced insulin's effect to 11-fold in both DF and control cells. However, DF cells were only 50% as responsive as normal fibroblasts to the mitogenic effect of 10% calf serum ($p < 0.001$).

Fig. 1. Effect of somatomedin, insulin, EGF, and serum on [³H]thymidine incorporation in DF and control fibroblasts. Human fibroblasts were plated in 35-mm dishes and grown to confluency. The cells were then serum-starved for 48 h, and the medium changed to the indicated experimental medium. [³H]thymidine incorporation was measured at 21–23 h. Results are the means $\pm$ SEM of three determinations expressed relative to SFM (which is assigned an arbitrary value of 1.0). [³H]thymidine incorporation values for SFM were 0.82 and 0.31 per 10^6 cells for DF and control (GM3652) cultures, respectively. SM, 50 ng/ml SM-C/IGF-I; insulin, 100 ng/ml; EGF, 5 ng/ml; HHS, 0.25% HHS; CS, calf serum, 10%. *indicates a significant difference between DF and control cells at $p < 0.001$.

Fig. 2. Effect of dexamethasone on somatomedin stimulation of [³H]thymidine incorporation. Fibroblasts were treated as described in Figure 1. Confluent cultures were serum starved with 10^{-7} M dexamethasone (stippled bars) or ethanol vehicle (solid bars) for 48 h and the medium then changed to the experimental medium. [³H]thymidine incorporation was measured at 21–23 h. Results are means $\pm$ SEM of three determinations expressed relative to SFM (which is assigned an arbitrary value of 1.0). [³H]thymidine incorporation values for SFM were 0.11, 0.48, and 0.47 per 10^6 cells for N1493, GM3652, and DF cultures, respectively. SM, 50 ng/ml SM-C/IGF-I; HHS, 0.25% human hypopituitary serum. *indicates a significant difference with dexamethasone at $p < 0.001$.

Effect of dexamethasone on somatomedin stimulation of [3 H]thymidine incorporation. Glucocorticoids have been shown to be necessary for optimal somatomedin stimulation of DNA synthesis in normal human fibroblasts *in vitro* (7). Thus, the interaction between SM and dexamethasone was investigated in DF fibroblasts (Fig. 2). In accordance with the results shown in Figure 1, SM (50 ng/ml SM-C/IGF-I) alone stimulated [3 H]thymidine incorporation 6-fold in DF and the two control cell lines. Furthermore, all three cell lines were responsive to the enhancing effect of HHS on SM action, with SM + 0.25% HHS stimulating [3 H]thymidine incorporation 25-, 28-, and 30-fold in N1493, GM3652, and DF cultures, respectively.

Dexamethasone (10^{-7} M) alone had no stimulatory effect on [3 H]thymidine incorporation in control or patient cells. However, preincubation of normal human fibroblasts lines N1493 and GM3652 with dexamethasone resulted in a dramatic synergistic increase in SM-stimulated [3 H]thymidine incorporation. In control cells pretreated with dexamethasone, SM (50 ng/ml SM-C/IGF-I) stimulated [3 H]thymidine incorporation 15- and 20-fold in SFM and 50- and 80-fold in the presence of 0.25% HHS. In contrast, preincubation of DF cells with dexamethasone had no effect on SM stimulation of [3 H]thymidine incorporation in either SFM or with 0.25% HHS. In this experiment, [3 H]thymidine incorporation was measured at 21–23 h, however, this lack of synergistic action between SM and dexamethasone was evident in DF fibroblasts even when [3 H]thymidine incorporation measurements were taken up to 32 h following SM stimulation (data not shown).

Effect of pure SM-C/IGF-I on the replication of human fibroblasts. The [3 H]thymidine incorporation experiments presented in Figures 1 and 2 were performed using a preparation of SM which was a mixture of both SM-C/IGF-I and IGF-II. Table 1

corroborates these results using a pure synthetic SM-C/IGF-I and includes data showing the effect of insulin and SM-C/IGF-I on replication of DF and control fibroblasts. SM-C/IGF-I (100 ng/ml) and insulin (100 ng/ml) increased cell number 42 and 33%, respectively, in control cultures. In the presence of 0.25% HHS, SM-C/IGF-I and insulin increased cell number 125 and 100%, respectively, in control cultures. DF fibroblasts were capable of responding to the mitogenic effects of SM-C/IGF-I and insulin and to express synergism between 0.25% HHS and SM-C/IGF-I and insulin. However, when cell number was expressed as percent of serum-free medium, the DF responsiveness appeared to be less than control cells, especially in the presence of 0.25% HHS which alone had only a 10% effect in DF cells compared to a 42% effect in control cells.

Dexamethasone alone had a slight inhibitory effect on cell replication. However, SM-C/IGF-I + dexamethasone increased control cell number 83% in SFM and 229% in the presence of 0.25% HHS. Dexamethasone also potentiated insulin's mitogenic effect in control cells. In contrast, dexamethasone had little effect on SM-C/IGF-I or insulin stimulation of DF cell replication.

Also shown in Table 1, the increase in cell number in response to serum was 58% less in DF as compared to control cultures.

DISCUSSION

Tokuhiro *et al.* (2) described patient DF as having growth retardation associated with severe insulin resistance. None of the usual causes, such as naturally occurring antibodies against insulin or the insulin receptor, abnormal forms of circulating insulin, or increased levels of insulin antagonists could be found to explain this unusual disorder. Insulin binding and insulin-stimulated glucose transport were normal. Circulating levels of SM-C/IGF-I, a biochemical relative of insulin, were found to be normal. The authors proposed an undefined postreceptor defect as the cause of the abnormal growth condition. The data presented herein suggest one possible mechanism for impaired cellular growth in DF.

Fibroblasts from DF appeared to have normal increases in [3 H]thymidine incorporation in response to SM-C/IGF-I, insulin, and EGF in SFM. DF and control cells also showed similar enhanced responsiveness to SM-C/IGF-I and insulin in the presence of 0.25% HHS. Therefore, the ability of SM-C/IGF-I, insulin, and EGF to stimulate DNA synthesis in DF fibroblasts was not consistent with a generalized defect in growth factor responsiveness in these cells.

When control fibroblasts were preincubated with dexamethasone and then stimulated with SM-C/IGF-I, both in SFM and in the presence of 0.25% HHS, there was a striking synergistic increase in [3 H]thymidine incorporation. Dexamethasone also potentiated SM-C/IGF-I-stimulated multiplication of control cells. In contrast, in DF cells, preincubation with dexamethasone produced little or no enhancement of SM-C/IGF-I stimulation of DNA synthesis or fibroblast replication. Similarly, insulin in the presence of dexamethasone produced a synergistic increase in cell number in control cells, while in DF cells this synergism was impaired.

The defect in DF fibroblast mitogenesis appears to be distal to the insulin and SM-C/IGF-I receptor and on a specific pathway leading to DNA synthesis which is common to the action of SM-C/IGF-I and insulin. Evidence that the defect is specific for DNA synthesis includes previous findings that glucocorticoids do not alter SM-C/IGF-I binding or SM-C/IGF-I stimulation of RNA and protein metabolism in normal human fibroblasts (1) and that insulin-stimulated glucose uptake was not altered in DF cells (2).

Both the experiments presented here and the study of Tokuhiro *et al.* (2) demonstrated a 40–60% decrease in [3 H]thymidine incorporation and replication of DF cells in response to serum.

Table 1. Effect of IGF-I, insulin, and dexamethasone on the replication of human fibroblasts*

	Cell no. ($\times 10^5$)	
	Control	DF
EtOH	0.24 (100)	0.40 (100)
DEX	0.20 (83)	0.34 (83)
SM-C/IGF-I	0.34 (142)	0.53 (131)
SM-C/IGF-I + DEX	0.44 (183)	0.55 (137)
Insulin	0.32 (133)	0.47 (117)
Insulin + DEX	0.40 (167)	0.51 (128)
HHS	0.34 (142)	0.44 (110)
HHS + DEX	0.29 (121)	0.41 (102)
SM-C/IGF-I + HHS	0.54 (225)	0.62 (156)
SM-C/IGF-I + HHS + DEX	0.79 (329)	0.65 (162)
Insulin + HHS	0.48 (200)	0.53 (131)
Insulin + HHS + DEX	0.59 (245)	0.57 (142)
10% calf serum	1.14 (475)	0.78 (195)

* Fibroblasts were plated sparsely in 2% calf serum. Cells were allowed 24 h to attach, washed, and the medium changed to the indicated experimental medium. The number in parenthesis is the cell number on day 3 expressed as the percent of cell number in SFM on day 3 (which has been assigned an arbitrary value of 100%). Results are the average of duplicate wells. Percent variation (difference between observations divided by the mean $\times 100$) was less than 10%.

EtOH, 0.2% ethanol; DEX, 10^{-7} dexamethasone; SM-C/IGF-I, 100 ng/ml; insulin, 100 ng/ml; HHS, 0.25%.

These observations with serum may be related, in part, to the interaction of SM-C/IGF-I and insulin with glucocorticoids in the regulation of fibroblast growth. The concentration of hydrocortisone in adult human serum ranges from 22–226 $\mu\text{g/liter}$ (60–600 nM). Parenthetically, subject DF had hydrocortisone levels within the normal range (2). Although in these experiments a concentration of dexamethasone was used which was found to produce a maximal effect (100 nM), earlier studies have shown a significant effect on SM-C/IGF-I stimulation of fibroblast replication with as little as 1 nM dexamethasone and 10 nM hydrocortisone (7). Physiological concentrations of glucocorticoids also have been shown to influence cell growth in other culture systems. The nature of the response, whether stimulatory or inhibitory, varies according to the cell type and culture conditions (13–15).

The present study was limited to fibroblast cultures. Nevertheless, this is the first report in which a postreceptor alteration in the interaction between SM-C/IGF-I or insulin and glucocorticoids may be linked to abnormal mitogenic action in a pathological growth state. On the other hand, the effects of SM-C/IGF-I and dexamethasone on DNA synthesis and replication of fibroblasts from DF share many similarities with the effects of these hormones on fibroblasts from aged individuals [donors 85–96 yr old (8, 12)]. Thus, in both situations, the response to SM-C/IGF-I alone and SM-C/IGF-I in the presence of 0.25% HHS was normal. However, there was a loss of synergism between SM-C/IGF-I and dexamethasone in promoting DNA synthesis and replication of fibroblasts from DF and old donors. The cellular mechanism for the mitogenic interplay between SM-C/IGF-I and glucocorticoids in human fibroblasts and the nature of the defect(s) in the regulatory scheme in insulin resistance, aging, and possibly other growth-deficient states are important research concerns currently under investigation.

Acknowledgments. The authors thank Dr. Heather Dean and Yvette Perry (University of Manitoba, Manitoba, Canada) for generously providing us with fibroblasts from patient DF.

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