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Stimulation of DNA Synthesis in Human Fibroblasts by a Human Nonsuppressible Insulin-like Protein (NSILP)[1]

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Summary

When nonsuppressible insulin-like protein (NSILP) isolated and purified from human serum was added at concentrations of 5 and 50 ug/ml to cultures of human dermal fibroblasts, both cell proliferation and DNA synthesis were enhanced. However, NSILP, 50 ug/ml, had no effect on glucose uptake. In contrast, insulin, 40 ng/ml (1.0 mU/ml), had no effect on cell proliferation or DNA synthesis, but stimulated glucose uptake. These observations suggest that human NSILP may play an important role in tissue repair or growth by enhancing fibroblast proliferation, but not a significant glucoregulatory role.

Introduction

In certain *in vitro* biologic systems, human serum can be demonstrated to exert insulin-like effects which cannot be abolished by the addition of insulin-specific antibodies (1,2). This activity of serum has been termed nonsuppressible insulin-like activity (3) and includes a small molecular weight form, soluble in acid-ethanol and designated NSILA-S, and a larger molecular weight component, precipitated by acid-ethanol, and called NSILA-P (4). Recently, Poffenbarger has isolated a large molecular weight insulin-like protein called NSILP which cannot be dissociated into a small molecular weight

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Abbreviations: NSILP, nonsuppressible insulin-like protein; MEM, Eagle's minimal essential medium; FBS, fetal bovine serum.

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NSILA by acid treatment (5). We have had the opportunity to test a highly purified human serum NSILP in a fibroblast culture established from a normal adult human donor.

Materials and Methods

Radioisotopes and Chemicals: Methyl-[^{3}H]thymidine (specific activity 6.7 Ci/mMole) and U-[^{14}C]D-glucose (specific activity 4.86 mCi/mMole) were from New England Nuclear. NTB-3 Nuclear Track Emulsion was from Eastman Kodak. Porcine insulin (glucagon-free, single peak, 25 uU/ng) was from Eli Lilly. NSILP was obtained from human serum (5), and the biologic activity of the sample used in these experiments was between 4.5-5.0 mU NSILA/mg protein, as determined in the fat cell insulin bioassay (1). The NSILP immunoassay (6) of the sample used revealed that NSILP accounted for about 25% of the preparation (250 ug NSILP/mg protein). The contaminating proteins were albumin, haptoglobin, and a trace of transferrin. These proteins were inactive in the bioassay and did not cross-react in the NSILP immunoassay (6). The biological specific activity of purified NSILP, assayed in the fat cell insulin bioassay, approximated 50 mU/mg (5).

Culture: A 4 mm diameter punch biopsy was obtained from the skin of the deltoid region in a normal 26 year old female donor without any known medical disorders. The biopsy specimen was divided into pieces approximately 1 mm across which were then placed into a plastic tissue culture dish (65 mm, Lux) using previously described culture conditions (7), except that 5% (v/v) fetal bovine serum (FBS, ISI Biologicals) was used. Experiments were performed on early to mid-passage cultures (7th to 17th passage), with each passage representing a 1:2 split (one population doubling).

Experimental Cultures: For determination of culture growth curves and incorporation of [^{3}H]thymidine into DNA, approximately 5 x 10^{4} cells were dispersed into each of up to 100 glass scintillation counting vials and assays performed as described previously (7). [^{3}H]Thymidine was added at 0.5-1.0 uCi/vial, and cells were incubated for 4 hours before incorporation of the isotope into DNA was measured.

For radioautographic analyses of nuclear labelling with [^{3}H]thymidine, cells were dispensed into 4-chambered tissue culture chamber slides (Lab-Tek) at 2 x 10^{4} cells/chamber, and incubated in medium containing [^{3}H]thymidine (0.1 uCi/ml) for 24 hours. Cultures were then rinsed with cold (4°C) isotonic saline, treated with absolute ethanol for 5 minutes, rinsed with cold (4°C) 5% trichloroacetic acid (TCA) once, incubated with cold 5% TCA for 30 minutes, rinsed twice more with cold 5% TCA, rinsed with distilled H_2O twice, air-dried, coated with NTB-3 emulsion, and after exposure for about a week, were developed and then stained with hematoxylin and eosin.

In all experiments, cells were initially grown in Eagle's minimal essential medium (MEM) supplemented with nonessential amino acids, neomycin (50 ug/ml), and 5% (v/v) FBS for 24 hours, and then in the experimental medium for the duration of the study (4 to 6 days). Experimental medium was either MEM supplemented as described or with FBS reduced to 0.5% (v/v), with or without NSILP or insulin.

For measurement of glucose uptake, cells were incubated with [^{14}C]D-glucose as described previously (8), except that a 20 minute incubation period with [^{14}C]glucose was used, and FBS 0.5% (v/v) was present.

Results were analyzed using the two-tailed Student's *t* test.

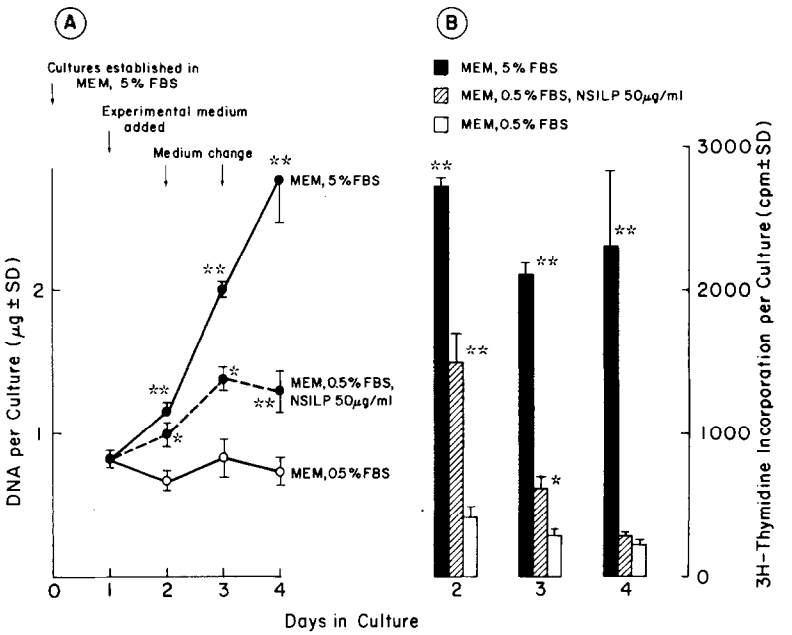

Figure 1: Passage 7 cells were grown in MEM, 5% FBS for 1 day, then in experimental medium for 3 days.
A. DNA per culture (mean±S.D. for triplicate cultures), * indicates $p<0.01$ and ** $p<0.001$ when compared to MEM, 0.5% FBS. In addition, on Days 3 and 4, $p<0.001$ when comparing MEM, 5% FBS with MEM, 0.5% FBS, NSILP 50 ug/ml, but no significant difference on Day 2.
B. [^{3}H]thymidine incorporation per culture (mean±S.D. for triplicate cultures), following 4-hour incubation with [^{3}H]thymidine on Days 2, 3, and 4. * indicates $p<0.01$ and ** $p<0.001$ when compared to MEM, 0.5% FBS.

Results

NSILP clearly stimulated both cell proliferation and DNA synthesis in adult human dermal fibroblast cultures (Figures 1 and 2). In the first experiment (Figure 1), cells were maintained in MEM and 5% (v/v) FBS immediately before the experimental medium was added. NSILP, 50 ug/ml, clearly enhanced both cell proliferation and DNA synthesis, but its effect was not as great as serum, and was not sustained. In the second experiment (Figure 2), cells were maintained in MEM and 0.5% (v/v) FBS for 2 days before experimental medium was added. A striking effect of NSILP on both cell proliferation and DNA synthesis was observed, especially with NSILP 50 ug/ml, but the effect was not sustained. In a third experiment, insulin had no effect on cell proliferation

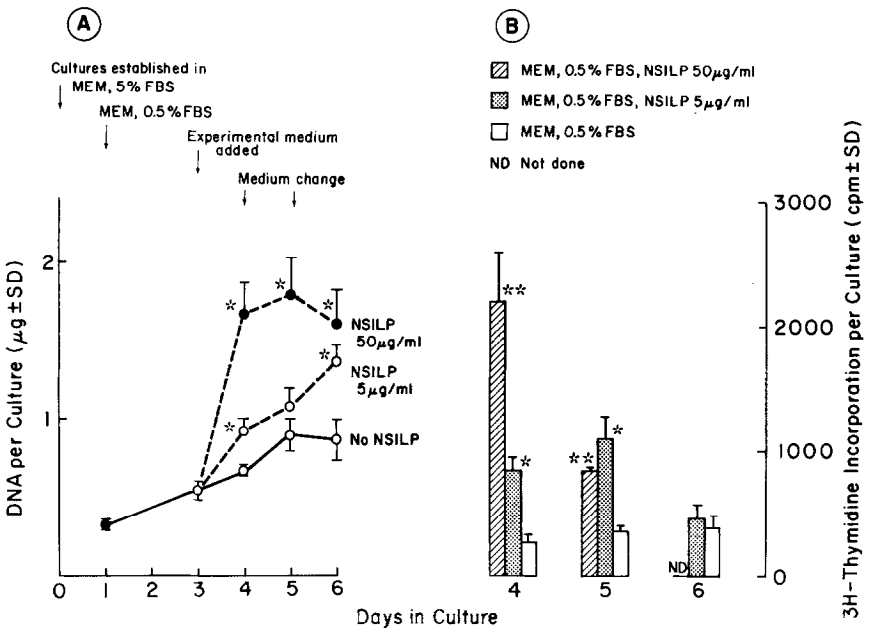

Figure 2: Passage 8 cells were grown in MEM, 5% FBS for 1 day, then in MEM, 0.5% FBS for 2 days, then in experimental medium for 3 days.
A. DNA per culture (mean±S.D. for triplicate cultures), * indicates $p<0.01$ when compared to MEM, 0.5% FBS. In addition, on Days 4 and 5, $p<0.01$ comparing NSILP 50 ug/ml with 5 ug/ml, but no significant difference on Day 6.
B. [^{3}H]Thymidine incorporation per culture (mean±S.D. for triplicate cultures), following 4-hour incubation with [^{3}H]thymidine on Days 4, 5, and 6. * indicates $p<0.01$ and ** $p<0.001$ when compared to MEM, 0.5% FBS.

(data not shown) but at a concentration of 400 ng/ml (10 mU/ml) did significantly enhance the rate of incorporation of [^{3}H]thymidine into DNA (Table 1). These results were confirmed by a radioautographic analysis of [^{3}H]thymidine incorporation into nuclei (Table 2). NSILP at 5 and 50 ug/ml increased the percent labelled nuclei observed; insulin at 4.0 and 40 ng/ml (0.1 and 1.0 mU/ml) was without effect. Finally, insulin at 40 ng/ml significantly increased glucose uptake, but NSILP 50 ug/ml had no effect (Table 3).

Discussion

Human serum NSILP has been extensively purified and partially characterized (5). It is a protein of 88,000 daltons, and is apparently synthesized by the liver (9). Its large size suggests that it would not readily enter the

Table 1

[^{3}H]Thymidine Incorporation per Culture (cpm±S.D.)

Conditions	Day 4	Day 5	Day 6
MEM, 5% FBS	10,870±220**	15,880±1410**	17,210±4880**
MEM, 0.5% FBS	1590±320	1140±180	1130±270
+Insulin 4.0 ng/ml	1810±280	1140±70	1000±180
+Insulin 40 ng/ml	2040±130	1480±200	1200±60
+Insulin 400 ng/ml	2630±80*	2550±100**	2200±150**

On day 0, cultures of passage 17 cells were established in MEM, 5% FBS. On Day 1, medium was changed to MEM, 0.5% FBS. On Day 3, medium was changed to experimental medium: MEM, 5% FBS; MEM, 0.5% FBS; MEM, 0.5% FBS + insulin (4.0, 40, or 400 ng/ml), with medium changes on days 4 and 5. On Days 4,5 and 6, [^{3}H]-thymidine incorporation per culture was determined following a 4-hour incubation with [^{3}H]thymidine. *indicates $p<0.05$ and ** $p<0.001$ when compared to MEM, 0.5% FBS.

Table 2

Percent [^{3}H]Thymidine-Labelled Nuclei (mean±S.D.)

Conditions	Day 3-4	Day 4-5
MEM, 0.5% FBS	14.7±2.0	16.2±3.2
+NSILP 5 ug/ml	28.2±2.8**	21.3±0.6
+NSILP 50 ug/ml	52.5±3.0***	30.0±4.8*
+Insulin 4.0 ng/ml	16.7±1.0	12.3±1.0
+Insulin 40 ng/ml	18.5±2.0	15.2±1.8

On Day -1, cultures of passage 7 cells were established in MEM, 0.5% FBS. On Day 0, medium was changed to MEM, 0.5% FBS, and on Day 3 to experimental medium: MEM, 0.5% FBS; MEM, 0.5% FBS + NSILP (5 or 50 ug/ml); MEM, 0.5% FBS + insulin (4.0 or 40 ng/ml). On Days 3-4 and 4-5, cells were labelled with [^{3}H]thymidine for 24 hours. Results are expressed as the mean of triplicate counts (1000 cells counted). * indicates $p<0.02$, ** $p<0.01$, and *** $p<0.001$ when compared to MEM, 0.5% FBS.

Table 3

Glucose Uptake Over 20 minutes (mean cpm±S.D.)

Conditions	Expt 1	Expt 2
MEM, 0.5% FBS	288±48	349±20
+NSILP 50 ug/ml	349±45	338±26
+Insulin 4.0 ng/ml	336±25	366±42
+Insulin 40 ng/ml	441±10**	455±46*

Confluent cultures were maintained in MEM, 0.5% FBS, and glucose 40 mg/dl for 24 hours. Cultures were then refed fresh medium of the same composition, preincubated in the presence of the above conditions for 60 minutes, then [^{14}C]D-glucose (0.5 uCi/ml) was added for 20 minutes. * indicates $p<0.05$ and ** $p<0.01$ when compared to MEM, 0.5% FBS.

extravascular space except when vascular integrity has been damaged, as with trauma. NSILP constitutes about 90% of the total NSILA assayed in the insulin bioassay (4,5). The normal serum levels of NSILP are 1-4 ug/ml, increasing with pregnancy, neoplasia, acromegaly, burns, and trauma, and decreasing with hypophysectomy, thyroidectomy, and starvation (9-11). Thus the observations reported here that physiologic concentrations of NSILP promote both cell proliferation and DNA synthesis in human fibroblast cultures support the concept that this protein may play an important role in tissue repair processes *in vivo*. Furthermore, serum NSILP does not change following oral glucose (unpublished), suggesting that it may not be a physiologically important glucoregulatory hormone. This is further supported by our finding that unlike insulin, it does not increase glucose uptake by human fibroblasts. Thus, NSILP and insulin exert different effects on fibroblast cultures, indicating that these two proteins are probably bound to different receptors.

In conclusion, our observations indicate that human NSILP may play an important role in tissue repair processes by enhancing fibroblast proliferation but appear not to have a significant glucoregulatory role. These findings in the fibroblast also support the previously reported conclusion from

receptor binding studies in another cell, the adipocyte, that NSILP receptors are distinct from insulin receptors (12). Finally, NSILP can be added to the growing group of mitogenic serum factors that are active in fibroblast cultures.

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