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Direct Mitogenic Effects of Human Somatomedin on Human Embryonic Lung Fibroblasts

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Summary

The ability of purified basic somatomedin to reinitiate cell division in nondividing cultures of human embryonic lung fibroblasts (WI-38) maintained in serum-free medium was determined in order to assess the direct mitogenic effect of this substance on mammalian tissue. Resting cultures were prepared by incubation of the cells in serum-free medium for 48-72 hours. Addition of partially purified somatomedin resulted in cellular hypertrophy, DNA synthesis and cell division with a time course similar to that seen when serum was added. Although cells divided in response to physiological concentrations of somatomedin, doses up to 100x in excess of this did not produce as much cell division as 10% fetal calf serum. Addition of fresh medium containing somatomedin to cells previously stimulated by somatomedin failed to induce further cell division. A highly purified somatomedin preparation also stimulated cell division.

Introduction

The importance of growth hormone (GH)[1]-dependent factors in regulating cell division has been demonstrated in studies which have shown that serum from GH-deficient patients is incapable of supporting normal cell division in both chick embryo and human fibroblasts in culture (1, 2). The mitogenic activity of these sera could be returned towards normal by treatment of the patients with GH but not by adding GH to the cultures.

Several peptides which mediate GH effects on cell growth and replication have been isolated in varying degrees of purity and their effects on cells in

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[1]Abbreviations used: SM-Somatomedin; IGF-insulin-like growth factor; MSA-multiplication stimulating activity; [^{3}H]TdR-[methyl-^{3}H]thymidine; FCS-fetal calf serum; PBS-phosphate buffered saline; TCA-trichloroacetic acid; RRSM-somatomedin activity as measured by radioreceptor assay.

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culture have been studied. SM-A, SM-C, IGF and MSA have all been shown to stimulate cell division in chick embryo fibroblasts (3, 4, 5, 6, 7) and [^{3}H]TdR incorporation in mammalian cells in culture (3, 8, 9). These substances, however, have been demonstrated to increase cell number in mammalian cells only in the presence of small amounts of serum protein (10, 8, 11) or when added with a variety of other hormones (12). To determine whether SM is by itself mitogenic, we have studied the ability of partially and highly purified basic SM, isolated from human plasma Cohn fraction IV-1, to stimulate cell division in resting diploid human cell cultures in the absence of serum or other added growth factor (s).

Materials and Methods

Cells

WI-38 cells were obtained from the American Type Culture Collection, Rockville, MD, and grown as monolayers in Ham's F12 medium supplemented with $NaHCO_3$ (14 mM), L-glutamine (to 2.5 mM) and 10% FCS (Grand Island Biological Company (GIBCO), Grand Island, New York) at 37°C in a 95% air-5% CO_2 atmosphere. All experiments utilized cells between the 25th and 35th population doubling level. Cells were detached from the surface for subculturing or counting by incubation in 0.05% Trypsin (GIBCO) in PBS (KCl 0.2 g/L, KH_2PO_4 0.2 gm/L; NaCl 8.0 gm/L; $Na_2HPO_4 \cdot 7\ H_2O$ 2.16 gm/L; pH 7.35) after washing in PBS.

Cells were counted using a particle counter (Coulter Electronics, Hialeah, FL) with a lower threshold setting of 395μ^3. Median cell volume was determined by determining the threshold setting which excluded 50% of the population. Thymidine incorporation was determined after adding [^{3}H]TdR 20 Ci/mMole (Radiochemical Centre, Amersham, England) to the cultures and incubating for various time intervals. At the end of incubation, the monolayers were washed three times with cold PBS and cold 10% TCA was added. The precipitate was washed with cold 5% TCA, dissolved in 5N NaOH and the radioactivity was counted in 10 ml of Bray's solution. Cell protein content was determined by the method of Lowry et al (13), on redissolved washed TCA precipitates using bovine serum albumin as reference standard.

Somatomedin

The SM used in these studies was purified from human plasma Cohn fraction IV-1 (kindly supplied by Connaught Laboratories, Ontario) as described by Bala and Bhaumick (14). During fractionation procedures, SM bioactivity was monitored with a previously described *in vitro* hypophysectomized rat cartilage bioassay (15) or radioreceptor SM assay utilizing human placental particulate membranes as described by Marshall *et al* (16). RRSM activity was calculated as described by Marshall *et al* (16) and expressed in terms of equivalent SM bioactivity units of the partially purified SM reference standard used in the RRSM assay. Briefly, after acid-ethanol-acetone extraction, the SM activity was sequentially fractionated on Sephadex G75 (1% formic acid), SP-Sephadex (C25), Sephadex G50 (1% formic acid), isoelectric focusing (pH 3-10, ampholytes in sucrose density gradient), dialysis (Spectropor 3000), and Sephadex G50. In these studies, the partially purified SM consisted of the peak SM activity eluted from SP Sephadex, indicated molecular size 14,000-6000 (from previous gel filtration step), containing approximately 290 SM bioactivity units and 450 RRSM units/mg protein and <2.5μUnits/mg radioimmunoassayable insulin. The highly purified (final step) SM showed a single band on SDS acrylamide gel electrophoresis, a molecular weight near 7500, a pI near 8.6, a preliminary amino acid sequence similarity of the first 5 N-terminal amino acids to IGF-I (17), an equipotent and parallel dose response curves to most highly purified SM-C (kindly proved by Dr. J.J. Van Wyk) and near 4000 units of SM bioactivity and RRSM per mg (15). Fetal calf serum contained 3 RRSM units/ml. Units of SM activity expressed in Results are RRSM units.

Results

Incubation of WI-38 cells in medium without serum for 48-72 hours resulted in a marked decline in proliferation so that no further cell division occurred after the initial 24 hours (data not shown). Readdition of serum to resting cultures (Fig. 1) was followed by a wave of thymidine incorporation into acid insoluble material which began after a lag of 12 hours and reached a peak at

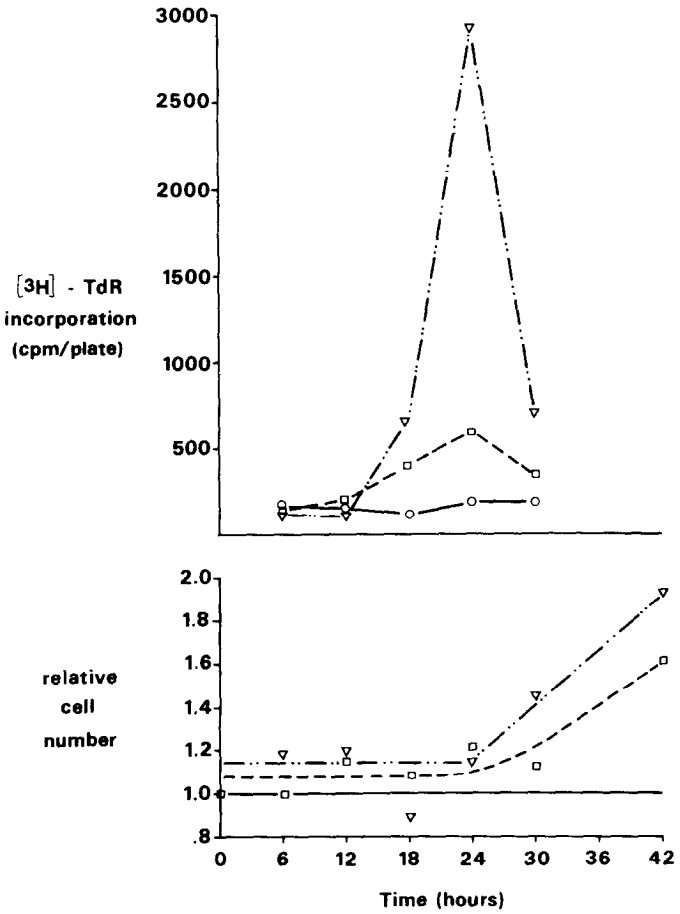

Fig. 1 Effect of 10% FCS or partially purified SM on thymidine incorporation and cell number. Cells were plated onto 35-mm tissue culture dishes in F12 + 10% FCS. Twenty-four hours later the plates were washed several times with PBS and incubated in F12 without serum for a further 48 hours to produce resting cultures. At that point (0 time), medium was removed from the plates and fresh medium with no addition (o——o), 50U/ml SM (□--□) or 10% FCS (▽-•••-▽) was added. Two hours before the indicated times, 5μCi [^{3}H] TdR was added to the appropriate plates. At the indicated times, triplicate plates with each addition were processed as described in Materials and Methods for cell number and thymidine incorporation. For each time point, relative cell number is the ratio of the cell number on the treated plates to the cell number on those plates continued in serum-free medium.

24 hours. Increases in cell number could be seen at 36 hours and by 42 hours 80% of the initial population had divided.

Addition of the partially purified SM instead of FCS to resting WI-38 cultures resulted in a significant increase in [^{3}H] TdR incorporation and cell number which followed the same time course as that seen after serum addition

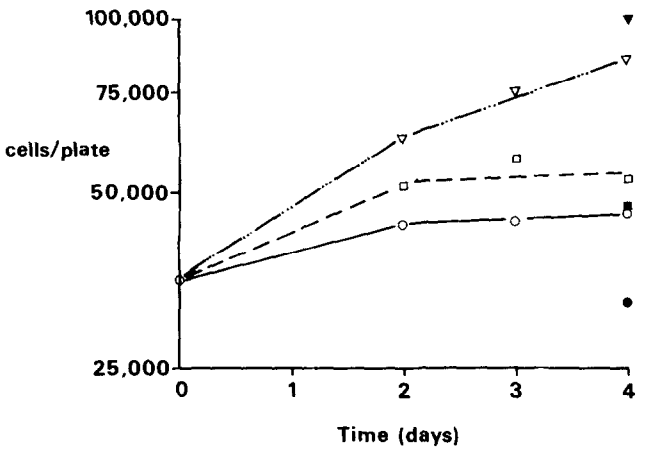

Fig. 2 Effect of partially purified SM or serum on prolonged cell division by resting cultures. Resting cultures were prepared as in Fig. 1. On day 0, fresh medium containing no serum (o——o), SM 16U/ml (□--□) or 10% FCS (▽···▽) was added. Cell number was determined at the indicated times. In addition, medium on some plates was changed on day 2, fresh medium with the same additions was added and cell number was determined 2 days later (indicated by shaded symbols).

(Fig. 1). In the presence of FCS, cells underwent several rounds of division (Fig. 2) but in the presence of partially purified SM instead of FCS, only a fraction of the population divided and no further division occurred after 48-72 hours. Addition of fresh medium with SM to cells so stimulated with SM resulted in no further increase in cell number (Fig. 2). Further addition of fresh medium containing 10% FCS instead of SM to these same cultures resulted in further cell division indicating that the cells could still respond to a mitogenic stimulus (data not shown).

The number of cells dividing in response to SM increased with increasing doses (Fig. 3). Significant increases were seen with doses within physiological concentrations (1 ml of human serum contains 1 unit of SM activity). Concentrations 100x greater failed to induce cell division in the whole population. Human serum albumin added over the same range of concentrations did not stimulate cell division (data not shown).

Cellular hypertrophy precedes DNA synthesis in cells stimulated by serum addition (18). As seen in Table 1, stimulation by partially purified SM also resulted

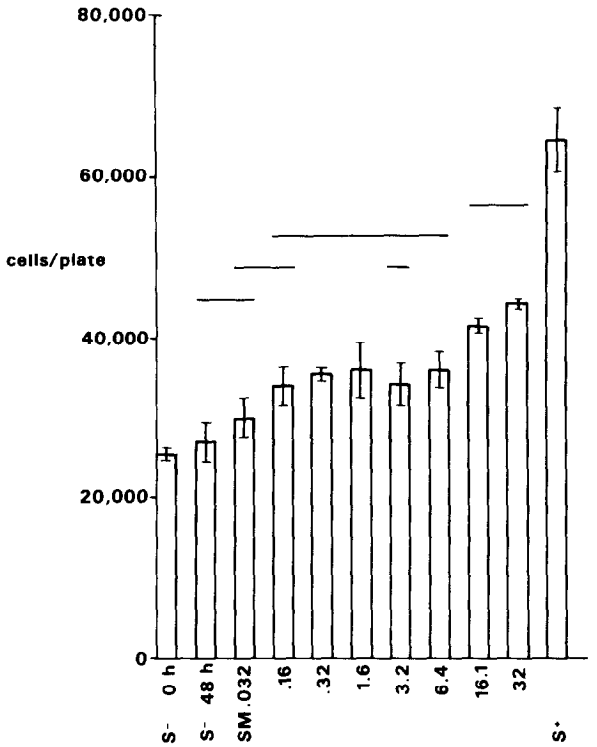

Fig. 3 Effect of increasing concentrations of partially purified SM on cell number in resting cultures. Resting cultures were prepared as in Fig. 1. At 0 time, cell number was determined and fresh medium with no serum, 10% FCS or the indicated concentrations of SM was added to the cells. Cell number was determined 48 hours later. Data are expressed as mean ± SEM. SM additions and S^- at 48 hours were compared with each other by one-way analysis of variance ($F=7.54$ $p<.005$). The means were then compared by Duncan's multiple range test. Means not significantly different from one another are overscored by lines at the same vertical level. (S^- 0 h, 48 h: 0 time and 48 hours later, fresh medium with no serum, SM. 032-32: SM concentration (U/ml); S+: medium + 10% FCS.

Table 1. Effect of partially purified SM on prereplicative cell growth.

Addition	Median Cell Volume (μ^3)†	Protein Content (μg/plate)†
Medium - no serum	1951 ± 72	8.75 ± .635
Partially purified SM (16.1 U/ml)	2322 ± 99*	14.9 ± 1.52*
Medium + 10% FCS	2503 ± 119*	15.1 ± 1.03*

† mean ± SEM
*different from no serum ($p<.05$)

Resting cultures were prepared as in Fig. 1 and then stimulated by changing medium to fresh medium with the indicated additions. Twenty-five hours later, cell volume and protein content were determined as described in Materials and Methods. Cell number was not different between groups at 25 hours and the mean cell number ± SEM was 25,740 ± 1506 cells/plate.

Table 2. Effect of highly purified SM on cell division.

Addition	Cells/plate (Mean ± SEM)
Medium-no serum	45,582 ± 1600
SM 30 U/ml	52,825 ± 2416*
SM 150 U/ml	56,600 ± 1591*
10% FCS	79,495 ± 685*

*different from $\bar{S}$: $p<.05$

Highly purified SM (described in text) was diluted in medium without serum and added directly to resting cultures prepared as in Fig. 1. In addition, some cultures received medium containing 10% FCS and some received serum-free medium instead of SM. Cell number was determined 48 hours later.

in a significant increase in median cell volume and cell protein concentration by 25 hours after addition (at the time of maximum rate of thymidine incorporation and preceding the increase in cell number).

Addition of highly purified SM to resting cultures in the absence of serum also significantly stimulated cell division (Table 2).

Discussion

These studies demonstrate that concentrations of SM in the physiological range are capable of stimulating cell division in serum-deprived non-dividing cultures. Only a fraction of the population of cells prepared in this way is sensitive to stimulation by SM. After the initial round of SM-stimulated division, cells become refractory to further SM stimulation. These data suggest that upon serum removal, cell progression through the cell division cycle is arrested at "restriction point" (19) where at least some of the cells are sensitive to stimulation by SM. The division cycle initiated by SM, however, leaves cells at a "restriction point" where they are no longer sensitive to SM but remain sensitive to serum. The observation that cycloheximide-treated cells can initiate DNA synthesis in response to serum but not in response to purified SM supports this (20) and suggests that sensitivity to SM might be restored by addition of other factors.

Evidence suggests that all mitogens activate a common program of cell growth which is then followed by DNA replication and cell division (21). Our results demonstrate that SM acts in a similar way, increasing cell protein content and

volume over the time preceding cell division. In this respect, the SM effects are similar to those of serum (18).

The mitogenic activity of serum is greater than that of plasma (22) and is contributed to by a number of factors not normally present in plasma *in vivo*. These include activated proteases (23, 24) and factors released from platelets upon activation (25). The SM activity of plasma and serum are similar, however. Thus, although SM cannot account for all the mitogenic activities of FCS (26) (medium with 10% FCS contains 0.3U RRSM/ml and yet induces much more cell division than an equivalent concentration of purified SM), SM may nevertheless be an important component of the circulating replication stimulus *in vivo*.

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