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THE RELATIVE CONTRIBUTION OF SOMATOMEDIN TO THE SERUM-STIMULATED GROWTH OF HUMAN FIBROBLASTS

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SUMMARY

Culture conditions were defined allowing to demonstrate a stimulatory effect of both serum-contained and purified Somatomedin activity on incorporation of [3H] thymidine and replication of cultured normal human fibroblasts. The use of dialyzed human serum in MEM medium supplemented by 0.2 mM serine offered the necessary and sufficient culture conditions. A significant difference between normal and hypopituitary patients sera was found in their effect on the rate of [3H] thymidine incorporation ($p < 0.0001$) and on cell replication ($p < 0.01$). Purified Somatomedin-C, in MEM without serum, is a poor mitogen. Its activity was strongly enhanced by the addition of 0.1 % dialyzed serum and 0.2 mM serine without, however, exceeding the stimulatory level of 1 % whole normal serum. The requirement of concomitant presence, for optimal in vitro cell growth, of different low and high MW serum components is discussed.

INTRODUCTION

Human fibroblasts in culture, as many other non-established cell-strains, require serum to grow. Several factors stimulating cell growth have been isolated from blood and animal tissues (1, 2). Among them are a group of Growth Hormone (GH) - dependent peptides with insulin-like biologic effects (3) – the Somatomedins (SM) (4, 5, 6) – some of which have been purified and demonstrated to enhance [3H] thymidine ([3H] Td) incorporation into fibroblasts (7). Their effect however was quite low as compared to that of serum.

Abbreviations : BSA, bovine serum albumin ; GH, growth hormone ; MEM, minimal Eagle's medium ; MSA, multiplication stimulating activity ; NSILA, non suppressible insulin-like activity ; SM, Somatomedin ; SM-C, Somatomedin-C ; [3H] Td, tritiated thymidine.

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In order to assess the extent of the contribution of the GH-dependent factors to the growth-stimulating serum activity, hypopituitary and normal individuals sera were compared in in vitro fibroblast growth assays. Moses et al. (8) found that [3H]Td incorporation into chick embryo fibroblasts was stimulated more by sera from GH-treated hypopituitary patients than by sera from untreated patients. They failed to find such effect in their assays on human fibroblasts. One hypothesis made to explain this discrepancy was to assume a difference between the two species in cell sensitivity to GH-dependent growth factors or in the control mechanism of DNA synthesis. However, in earlier experiments, Van Wyk et al. (9), using partially purified preparations of SM-C, observed an increase in [3H]Td incorporation into human fibroblasts. The aim of the present study was to define culture conditions in which the response of human fibroblasts to the human serum SM activity becomes reproducibly detectable and to compare the stimulatory capacity of hypopituitary and normal human sera and of purified SM-C.

MATERIALS AND METHODS

Materials : L-serine (Merck) and bovine serum albumin(BSA) (Calbiochem) are>99 % pure. Somatomedin-C (SM-C) is a generous gift of Pr. J.J. Van Wyk (Chapel Hill-NC-USA). The preparation used in this study was a fraction obtained during the final purification of SM-C. It contains approximately 2000 SM-C units per mg protein as measured by radioimmunoassay (RIA). Human growth hormone (France Hypophyse) is 95 % pure.

Cells : Fibroblasts were derived from a newborn foreskin biopsy. The cells are cultured in MEM medium with Earle's salts, supplemented with 10 % decomplemented fetal calf serum, in a 95 % air + 5 % CO_2 atmosphere. They were used between the 15th and the 20th generation.

Cell multiplication assay : Sparse cultures (2500 to 4000 cells / cm2) were obtained by seeding 100 000 cells in Falcon F-30 culture flasks in 5 ml of MEM and 10 % horse serum. After a 5 hour incubation at 37°C, the medium was discarded, the cells washed once and 5 ml of MEM plus 10 % test serum was added. At intervals of 2, 4 or 7 days the cells were trypsin dissociated and counted. The cultures remained sub-confluent during the whole course of the experiments. All assays were done in duplicates and 4 counts per sample were performed.

[3H] thymidine incorporation assay : 10 000 cells in 0.2 ml of MEM plus 10 % fetal calf serum were introduced into each well of a microtiter plate (Falcon 3040) to obtain a subconfluent density (25 000 cells / cm2). After 24 hours at 37°C the cells were washed once, and 0.2 ml of MEM without serum was added. A stable basal level of incorporation was reached within 18 hours. The mitogenic substances to be tested were then added (0-h) in 50 µl per well. [3H]Td (0.1 µCi per well, 30-50 Ci / mM, CEA, France) was introduced on the 8th hour and left until the 30th hour, which corresponds to one whole cycle of incorporation in the presence of human serum. The incorporation was stopped by aspiration of the medium. The cells were harvested on a cotton swab, washed in 3 successive baths of 10 % TCA, 0.5 % TCA and in 95 % alcohol, then dissolved in 0.2 ml of Soluene-100 (Packard). Each assay was done in triplicate. When purified SM-C was tested, 0.5 % pure BSA was added to the medium, it was examined and found to have negligible effect on [3H] Td incorporation. It must be stressed that under the conditions described, the cells do undergo division but confluency is not reached. Statistical analysis was carried out according to the Mann and Whitney test for non parametrical populations (10).

Sera : Normal sera were obtained from young healthy men. Their Somatomedin content was close to 1 U / ml using a biological assay (11). The sera SM-C assayed by RIA (12) ranged from 1.12 to 2.76 U / ml. Hypopituitary sera were obtained from children with documented pituitary dwarfism. Their Somatomedin content was 0.09 to 0.60 U / ml using the bioassay and 0.02 to 0.15 U / ml in sera assayed by RIA.

Dialysis of serum was done three times against 50 volumes of MEM, for 24 hours, in Spectrapor-3 tubing (exclusion limit of 3500 MW) boiled before use for 30 min in distilled water containing 10 mM sodium EDTA and 100 mM $NaHCO_3$.

RESULTS

In agreement with previous findings (8, 13), our preliminary experiments showed no significant difference in the capacity to stimulate [3H]Td incorporation into cultured human fibroblasts when whole sera of 2 normal subjects and 4 hypopituitary dwarfs were compared. This led us to examine the effect of dialysis separated serum fractions. As shown in Fig. 1, the withdrawal by Spectrapor-3 dialysis of small molecular size (MW < 3500) serum components induced a decrease in [3H] Td incorporation. The recombined fractions had an activity that was equal to that of non dialyzed serum and higher than the arithmetical sum of the separately tested retained and excluded fractions. This appearance of synergy between high and low molecular

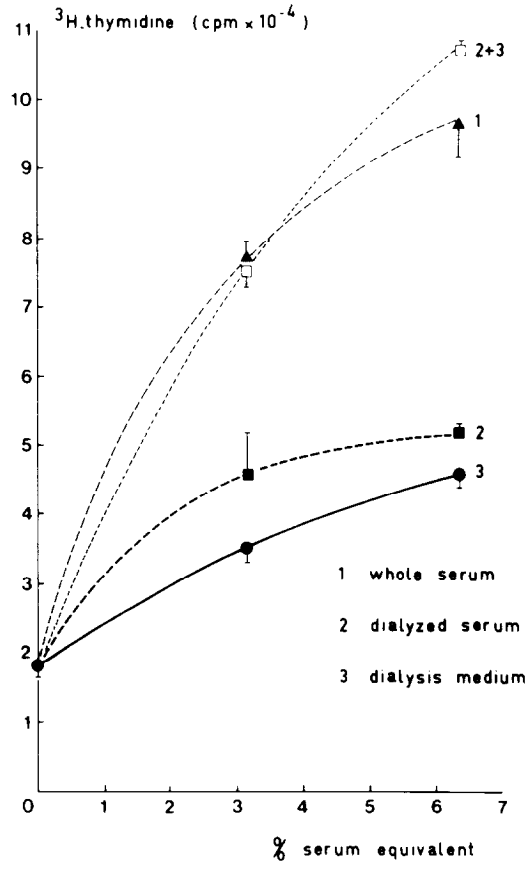

Fig. 1. The effect on [3H]Td incorporation into human fibroblasts of low and high MW, dialysis separated, serum fractions. The dialysis and the incorporation assays were done in presence of 0.25 % BSA. The % serum equivalent of the dialyzed serum (retained fraction) and of the dialysis medium (excluded fraction) are based on the initial serum volume.

weight serum components led us to examine the influence of serine (14) ; the MEM medium used in this study does not contain serine.

Data in Fig. 2 show that serine, when added to MEM without serum or with 1 % dialyzed normal human serum, had little effect on [3H]Td incorporation (Fig. 2a). A synergistic effect was found, however, with 5 % dialyzed serum, a maximum increase of 240 % being observed at 0.2 mM. Moreover, the addition of 0.2 mM serine to dialyzed serum brought the stimulation almost to the level obtained with whole normal serum (Fig. 2b). To determine

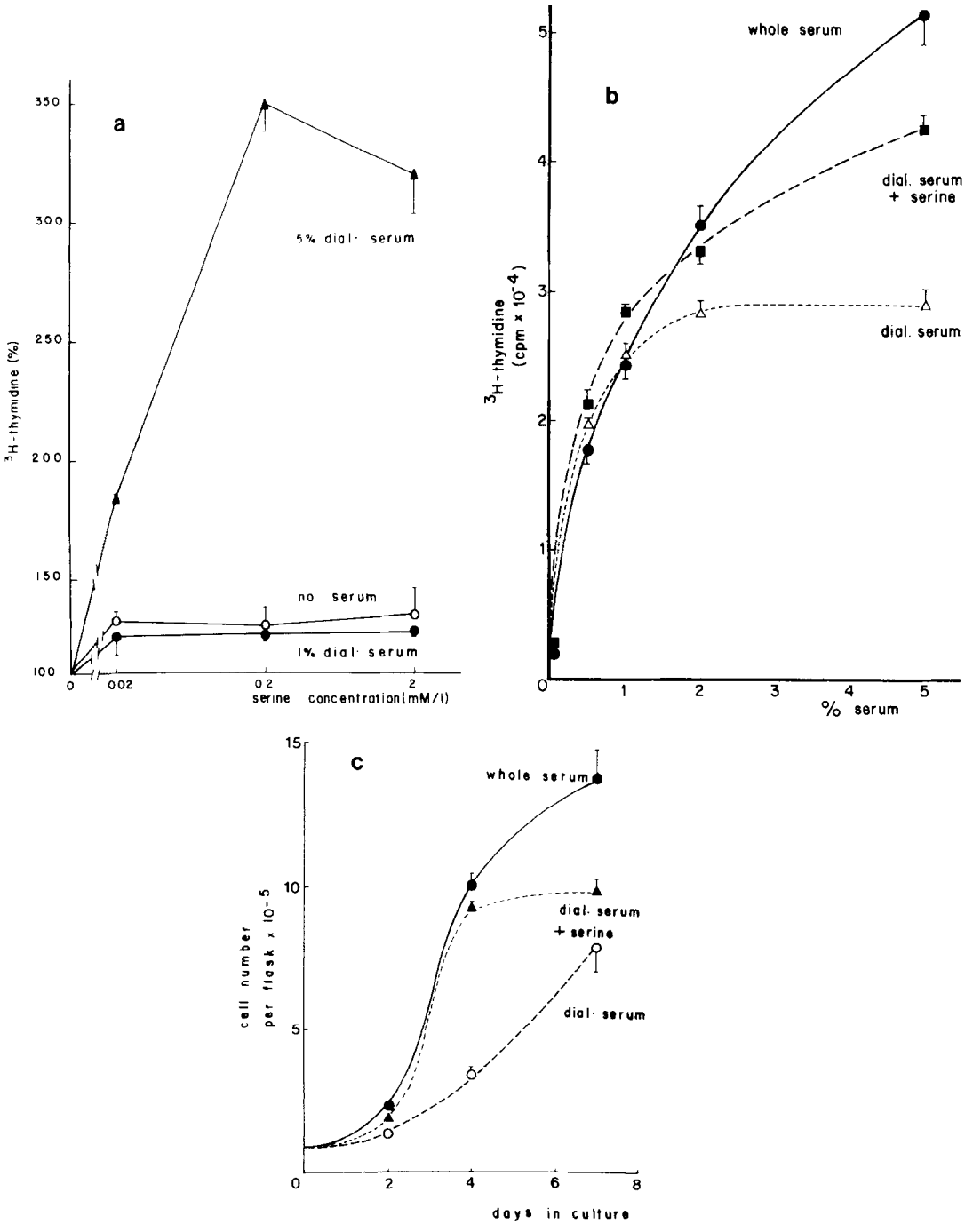

Fig. 2. The cooperative effect of serine and dialyzed serum on the growth of human fibroblasts. 2A - The effect of serine concentration on [3H] Td incorporation by cells grown in MEM without serum, with 1 % or 5 % dialyzed normal serum. 2B - [3H] Td incorporation stimulated by 0.2 mM serine added to dialyzed serum ; effect of serum concentration. 2C - The influence on fibroblast replication of 0.2 mM serine added to dialyzed serum. Cells were cultured in 10 % serum for 7 days without change of medium.

whether this effect of serine on DNA synthesis reflects an actual increase in fibroblast growth, cell counts were performed over a 7-day culture period (Fig. 2c). The overall growth rate of human fibroblasts is quite similar over the first 4 days of culture, whether the cells are grown in whole normal serum or in serine supplemented dialyzed serum. After 4 days the growth curves diverge : it levels off in the latter case and continues to mount in the former. The growth in dialyzed serum alone is consistently lower.

When 11 sera from normal subjects and 15 sera from hypopituitary dwarfs were examined under conditions as for Fig. 2b the following results were obtained. In presence of dialyzed sera supplemented with 0.2 mM serine there is a very significant ($p < 0.0001$) difference in the growth stimulating capacity of normal dialyzed sera versus sera of hypopituitary patients, the latter being less potent. Fig. 3 shows the mean and SEM values of [3H]Td incorporation assays obtained in 4 different experiments. The incorporation levels are expressed as percent of the basal level for each experiment. A better differentiation is seen at the higher serum concentrations as was the case for the serine stimulated thymidine incorporation (Fig. 2a). Human GH, when added directly to the medium at concentration of 10 ng to 5 μg/ml, had no effect on [3H]Td incorporation into fibroblasts. Neither the use of whole sera nor the dialyzed sera without added serine allowed the distinction between normal and hypopituitary sera (data not shown).

To study the effect of normal and hypopituitary sera over several days of culture, cell multiplication assays were performed over 4 days. Results of a representative experiment are shown in Fig. 4. In presence of the serum of a normal subject,

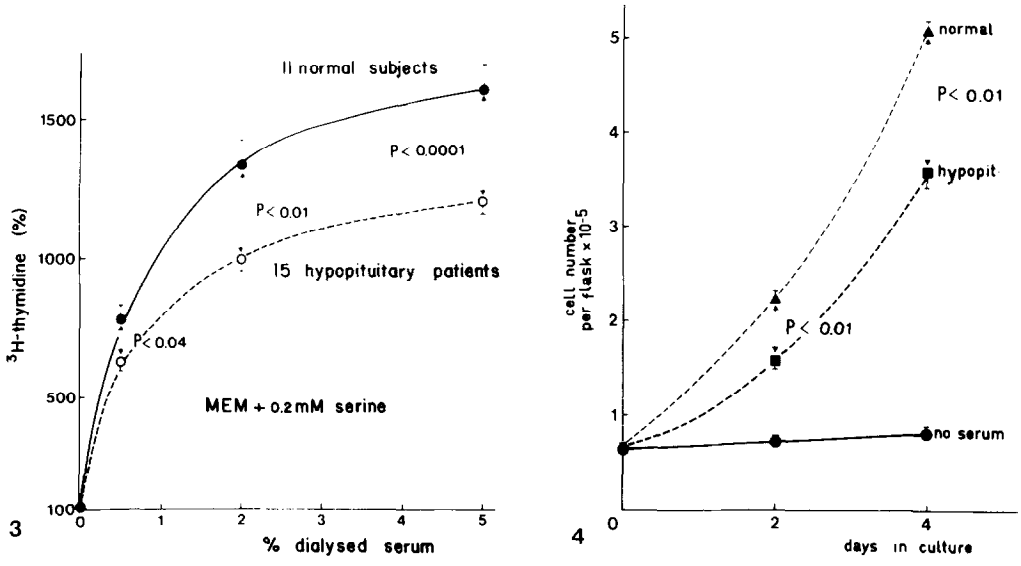

Fig. 3. Comparison of DNA-synthesis stimulatory activities of sera from normal subjects and from hypopituitary patients. The data are the combined means of 4 different experiments. The [3H] Td incorporation levels are expressed as % cpm obtained in cells grown in MEM only.

Fig. 4. Replication of human fibroblasts in presence of 10 % of a normal or hypopituitary patients serum.

there was an 8 fold increase in the number of cells over the 4 days of culture. In three separate experiments cell growth rate was significantly ($p < 0.01$) lower with the hypopituitary serum. Thus in contrast to the aforementioned [3H] Td incorporation study, here a decrease in the response of fibroblasts to hypopituitary patients sera could be demonstrated with identical results whether whole or dialyzed serum supplemented with 0.2 mM serine was used.

The effect of purified SM-C on [3H] Td incorporation was examined as shown in Fig. 5. When purified SM-C was the only factor added to the medium, it enhanced [3H] Td incorporation, reaching a plateau as of 10 ng/ml. This stimulation remained however very low as compared to that of normal whole serum. Addition of 0.2 mM serine produced a small but significant

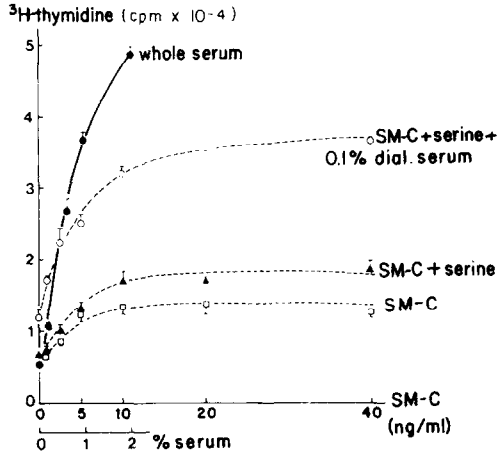

Fig. 5. SM-C stimulated [3H] Td incorporation ; the effect of 0.2 M serine and normal dialyzed serum. The serum contained 1.12 units SM-C per ml. Purified and serum SM-C were assayed by radioimmunoassay : 1 unit SM-C corresponds to 89 % of the serum employed and to 530 ng of the purified preparation. The 2 abscissae scales are drawn to correspond to the same SM-C units. All incorporations were performed in presence of 0.5 % BSA.

increase over the SM-C stimulation. In presence of 0.1 % dialysed normal human serum an increase, which cannot be attributed to the dialyzed serum SM-C content (0.001 U / ml as measured by the radioimmunoassay), was observed. The maximum of activity was reached at 20-40 ng / ml (0.04-0.08 U / ml) and corresponds to that obtained with only 1 % whole normal serum. The stimulation ratio here is about 3 fold over the basal level.

DISCUSSION

In the study presented, culture conditions were defined under which the mitogenic response of normal human fibroblasts to GH-dependent serum factor(s) becomes readily detectable. The factor(s) involved is different from growth hormone itself. It most probably is Somatomedin(s) as strongly indicated by (i) the significantly lower cell numbers obtained after 4 days of culture in presence of hypopituitary patients' sera versus normal controls ; (ii) by the lower short-term [3H] Td incorporation

into DNA of serum starved, sparsely seeded fibroblasts, grown in the hypopituitarys' sera under appropriate culture conditions ; (iii) the enhanced mitogenic effect of purified SM-C under the same conditions. For the demonstration of the two latter points, dialyzed sera and serine (or pyruvate) supplemented MEM were necessary. Purified SM-C is only a poor promoter of DNA synthesis when added to a chemically defined medium supplemented by pure BSA and, as we as well as others (8, 13) found, sera of GH deficient and of normal donors are equipotent in stimulating [3H]Td incorporation under standard culture conditions. Thus for the positive demonstration of a difference in serum capacity to stimulate in quiescent fibroblasts the transition to S-phase attributable to GH-dependent factors, additional low and high MW serum components are necessary. That serine might be a major one of the former was suggested by earlier work in which Eagle (14) and Elmore and Swift (15), on one hand, provided evidence for the importance of serine for the growth of human fibroblasts. On the other hand, Koumans and Daughaday (16) noted that serine is needed for the Sulphation Factor stimulated incorporation of ^{35}S into cartilage, later recognized as one of several activities of Somatomedins concerned with growth of both skeletal and nonskeletal tissues (4). Therefore it seems that serine is needed for at least two of the Somatomedin functions. Its specific role is yet to be elucidated.

The fact that the mitogenic effect of purified SM-C is considerably enhanced by the addition of as little as 0.1 % of dialyzed serum, as well as the sustained, though reduced, cell multiplication in presence of dialyzed Somatomedin-poor serum, indicates that other nondialyzable factors are involved. These observations concord well with the current views on cooperative

effects of a number of growth factors in the induction and stimulation of cell division related events (17). Notable in this context are the findings of Rechler et al. who observed an additive or more than additive effect of serum on the stimulation of [3H]Td incorporation into human fibroblasts by two other members of the SM family of factors - the NSILA and MSA (7). Also, in a study conducted in parallel with ours, Stiles et al. (17) demonstrated in the established mouse cell line Balb/c-3T3 that the platelet derived growth factor PDGF, the SM-C and hypopituitarys' serum are successively required for the cell commitment and expression of DNA synthesis. Whether the synergy between purified SM-C and the dialyzed serum, described in this report for normal human fibroblasts, is due to the presence in serum of platelet factors would now be of interest to find out. Another possibility, though not precluding the former, is that what the dialyzed serum supplies here in the way of high MW factors is a specific carrier protein. Indeed Van Wyk et al. (6) have shown that > 98 % of the serum SM is bound to ≥ 160 000 MW proteins, an association on the existence of which apparently depends the serum half-life of SM activity (18). Thus the fact that, in absence of other external protein than BSA, we observed with 40 ng/ml of SM-C (equivalent to SM-C content of about 8 % normal serum) a level of stimulation corresponding to that obtained with less than 1 % normal serum, is perhaps not that surprizing. The possible production of SM by cultured human fibroblasts (19) could reduce the effect of an exogenous SM. The assays described offer a relatively simple system in which the different hypotheses can be tested on non-transformed human cells.

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