A growth inhibitor implicated in the growth arrest of human fibroblasts

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Abstract When quiescent human fibroblasts are induced to divide, the sugar composition of a growth inhibitory glycoprotein is modified. The kinetics of the incorporation of glucosamine in the growth inhibitor follows the kinetics of cell growth. It increases as cells approach quiescence and declines when cells initiate proliferation. The results suggest that the modification of the sugar composition of this glycoprotein is coupled with the variation of its inhibitory potential and this way to the initiation and the arrest of cell division.

Key words: Human fibroblast; Growth inhibitor; Negative growth factor; Growth regulation

1. Introduction

Several growth inhibitors have been recently isolated and later purified (for a review see ref. [1]). They vary widely in structure and molecular weight, and thus in the mechanism of action.

The commitment to divide depends inter alia upon the balance between inhibitors and growth promotors. In general they accomplish their role through an increase in their concentration overriding the effect of their opposite, the growth factors.

We have isolated a growth inhibitor from human fibroblasts whose molecular weight and relationship with the division cycle are different from other inhibitors previously described [2]. We report herein the purification of this growth inhibitor that seems to exert its effect through a modification of its sugar composition.

2. Materials and methods

The cells used were the ICIG-7 human embryonic lung fibroblasts [3] maintained in Eagle's MEM supplemented with 10% foetal calf serum without antibiotics. They were routinely checked for mycoplasma contamination by nucleic acid hybridization with a commercial probe for mycoplasma DNA.

DNA synthesis was measured adding to the cultures [^{3}H]TdR with a specific activity of 2 Ci/mM and a final concentration of 0.1 μCi/ml. The nutrient medium was removed, the cultures were washed with medium without serum, and 2 ml of cold 10% trichloro acetic acid (TCA) were added during 5 min. The monolayer was rinsed with water and 500 μl of 0.3 M NaOH with 1% SDS were added. After 30 min this supernatant was added to 10 ml scintillation fluid (Readygel) with 500 μl of water and the radioactivity counted.

To extract the inhibitor, the cultures were labeled with [^{3}H]glucosamine (specific activity of 25 Ci/mM and final concentration of 1 μCi/ml) during the times indicated in the respective experiments and rinsed with 10 ml of 0.15 M NaCl three times and then with 10 ml phosphate-buffer saline (PBS) containing 0.1 M EGTA, pH 7.2. The cultures were then rinsed another time during 5 min with 5 ml of the same buffer. Two ml water was added and the material left attached to the substratum was removed with a cell scraper and filtered through Millipore 0.22 μm filters. The total cell extract was then passed through a Diaflo 5000 membrane (Amicon) and used for affinity chromatography running with water through a column of Sepharose-bound concanavelin A (Con A). The bound radioactivity was chromatographed with FPLC through polyacrylamide Bio Gel P4 (Bio-Rad) that separates below 4,000 Daltons, eluting with 15 mM NaCl at a rate of 10 ml/h, and finally with FPLC through polyacrylamide Bio Gel P2 (Bio Rad) with a fractionation range of 1,800–100 Daltons, eluting with water at the same rate. The columns for both chromatog-

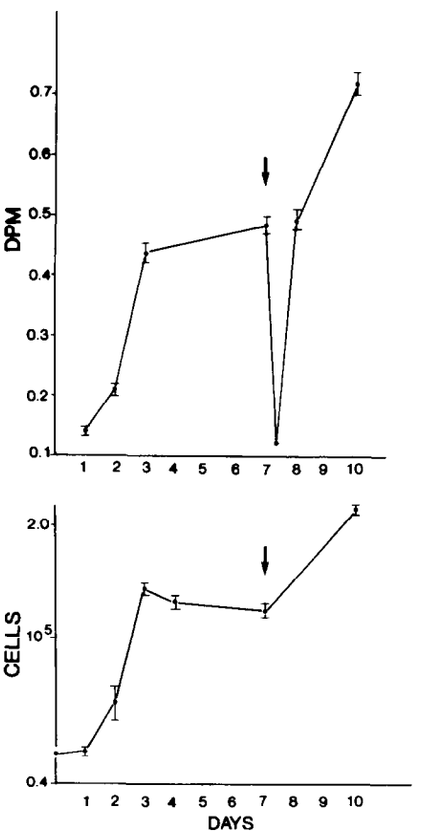

Fig. 1. Growth curve (below) and radioactivity from [^{3}H]glucosamine incorporated per cell (above). The arrow indicates the time when fresh nutrient medium was added to resting phase cultures.

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raphics had a length of 22 cm and a diameter of 1.6 cm (V_o = 13 ml, V_t = 31 ml). Purification was checked running the radioactive fraction obtained after the last purification step, through capillary electrophoresis [4]. The fractions corresponding to those containing the radioactivity that declined in stimulated cultures were used to test the growth inhibitory activity. [^{14}C]Galactose was used with a specific activity of 0.1 μCi in a final concentration of 1 μCi/ml.

A Superose 12 column (Pharmacia) was used with FPLC; 200 μl of the purified material were injected into the column, which had a V_o of 6 ml and a V_t of 30 ml. Elution was made with 0.15 M NaCl at a rate of 0.5 ml/min.

3. Results

Fig. 1 illustrates the number of cells and the [^{3}H]glucosamine incorporated during 1 h in the cell extract, at different days after seeding the cells and after a medium renewal at resting stage. The cells reached confluency 3 days after seeding. On the 4th day of the plateau of the growth curve, the nutrient medium was renewed and the cell number increased as expected. The glucosamine incorporation increased up to the 3rd day after subcultivation, remained stable during the plateau of the growth curve, decreased significantly 20 min after stimulation of the quiescent cultures, and increased later in parallel with the cell density.

Fractionation with capillary electrophoresis showed that the radioactivity that declined after stimulating the cells, was confined to one peak.

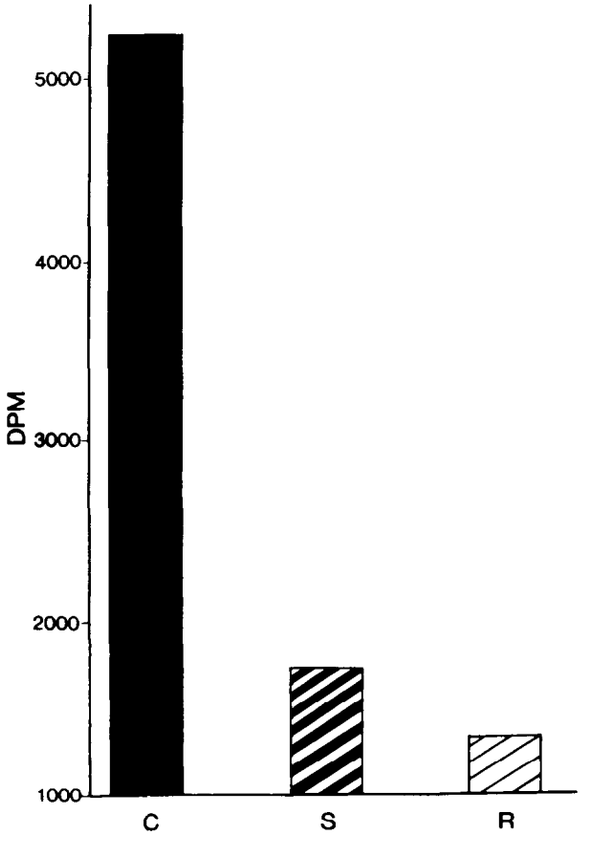

Fig. 2. Radioactivity from [^{3}H]TdR found after a 24 h labeling period of cultures grown without (C) and in the presence of purified extract from resting (R) and stimulated (S) cells.

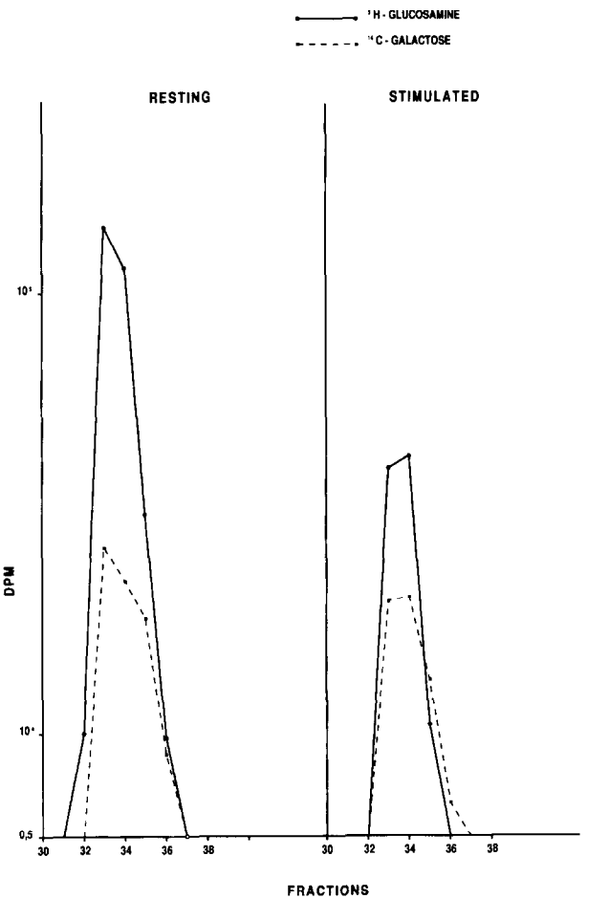

Fig. 3. Radioactivity from [^{3}H]glucosamine and [^{14}]galactose found in the material extracted from resting and stimulated cultures run through Superose 12.

The material purified from resting and stimulated cells, 1 h after stimulation, was added to sparse cultures in microplates 1 h after plating the cells and DNA synthetic activity was measured after a 24 h labeling period with [^{3}H]TdR. Cultures that received the same amount of fluid gone through the same procedures without cell extract, were used as controls (Fig. 2). The thymidine incorporated in the cultures treated with the purified extract was much less than that found in the controls. The molecule obtained from resting cells was more inhibitory than that obtained from stimulated cells ($t < 0.02$).

Resting and stimulated cells were double-labeled with [^{3}H]glucosamine and [^{14}C]galactose and the purified extract was run through Sepharose 12 (Fig. 3). The radioactivity from ^{3}H was significantly more decreased than that of ^{14}C.

4. Discussion

We have previously reported the separation of a cell fraction where the incorporation of glucosamine declined when growth factors were added to quiescent human fibroblasts [2]. This response was present during the entire cell proliferative life

span and was canceled when the cell population entered the terminal postmitotic stage and did not respond to growth factors. The cell fraction where the glycosamine incorporation declined was growth inhibitory.

As shown herein, the incorporation of glucosamine in the growth inhibitory molecule, is low when the cells are sparse, increases as the cells approach quiescence, and decreases when quiescent cells are stimulated. On the other hand, while the incorporation of glucosamine decreases when cells initiate proliferation, that of galactose varies little.

The purified fraction obtained from quiescent cells is more inhibitory than that obtained from stimulated cells 1 h after stimulation. It seems reasonable to conclude that the inhibitory effect is obtained through a change in its sugar composition.

The molecular weight of the inhibitory glycoprotein is less than 1,800 Daltons, hence this molecule is different from other growth inhibitors previously reported in regard to its molecular weight, relationship to the division cycle, and mechanism of action [1].

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