Increased hyaluronate synthesis is required for fibroblast detachment and mitosis

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Human-embryo fibroblasts were synchronized by means of colchicine and cytochalasin, and the production of hyaluronate was determined by [³H]glucosamine incorporation and ion-exchange chromatography. Cells arrested by colchicine synthesized small amounts of hyaluronate, whereas cells blocked by cytochalasin were stimulated in hyaluronate production. When the colchicine block was released, there was an increased synthesis of hyaluronate, which appeared first in the cellular fraction and was then shed into the culture medium. After release of the cytochalasin block, the hyaluronate production declined to that found with unsynchronized cells. A comparable increase of hyaluronate synthase activity was observed during mitosis. When hyaluronate synthesis was blocked by periodate-oxidized UDP-glucuronic acid, the cells were arrested in mitosis before rounding of cells. These results suggest that hyaluronate synthesis is required for detachment and rounding of cells during mitosis.

INTRODUCTION

Hyaluronate synthesis has been related to many cellular functions, including migration (Toole, 1972), invasion (Toole *et al.*, 1979; Knudson *et al.*, 1984), differentiation (Prehm, 1980), adhesion (Atherly *et al.*, 1976; Barnhart *et al.*, 1979), transformation (Hopwood & Dorfman, 1977) and cell proliferation (Tomida *et al.*, 1975; Hronowski & Anastasiades, 1980; Matuoka *et al.*, 1985). This information has been obtained mainly with cells differing in hyaluronate synthesis or by treating cells with hyaluronidase. Now we have developed inhibitors of hyaluronate synthesis that can be introduced into living cells and thus allow study of its functions (Prehm, 1985). Hyaluronate is synthesized at plasma membranes by alternate addition of UDP-hyaluronate to the substrates UDP-glucuronic acid and UDP-*N*-acetylglucosamine (Prehm, 1983*a,b*). Nascent chains are extruded through the membranes into the extracellular matrix (Prehm, 1984).

The successful assignment of biochemical processes to specific phases of the cell cycle depends in large part on the method of cell synchronization, because synchrony is rapidly lost. In the present study we used colchicine and cytochalasin to arrest human fibroblasts at different stages of mitosis, in order to investigate changes in glycosaminoglycan composition during mitotic detachment. We report that hyaluronate synthesis and synthase activity were stimulated during cell rounding in mitosis and that its inhibition arrested cell division.

MATERIALS AND METHODS

Cells

Human-embryo fibroblasts (HE-19) were used for the experiments. Determination of hyaluronate synthase activity (Prehm, 1980), preparation of periodate-oxidized UDP-glucuronic acid and its introduction into living cells by hypo-osmotic lysis of pinocytotic vesicles

(Prehm, 1985) have been described previously. Cells were blocked by addition of colchicine (0.01 µg/ml) or cytochalasin (2.0 µg/ml) to the growth medium before culture for 40 h. Cell synchrony was determined as described previously (Scott *et al.*, 1956).

Determination of glucosaminoglycan production

Human fibroblasts (3×10^5 cells) were grown in 5 ml of Dulbecco's modified Eagle's medium (GIBCO Europe) containing 5% (v/v) foetal-calf serum, streptomycin + penicillin (100 units of each/ml) and kanamycin (100 µg/ml) on 25 cm² dishes in the presence of 8 µCi of [³H]glucosamine/ml. Colchicine (0.01 µg/ml) or cytochalasin (2.0 µg/ml) was introduced into the culture for 40 h to block the cell cycle. After 40 h the medium was changed to normal growth medium. Cells and medium were harvested separately at different times of cultivation and the radioactivity incorporated into glycosaminoglycans was determined.

The cells were harvested by trypsinization or with the aid of a rubber 'policeman' in phosphate-buffered saline (0.01 M-NaCl/0.14 M-sodium phosphate buffer, pH 7.2), and the number of cells was determined with a haemocytometer. The cells were sedimented at 1000 *g* for 5 min and then suspended in 10 ml of phosphate-buffered saline. To determine hyaluronate synthesis, the labelled cells were kept at 100 °C for 10 min. After cooling to room temperature the cells were lysed by addition of 100 µl of Emulphogen BC-720 and digested with Pronase (1 mg/ml) for 24 h at 37 °C. The suspension was diluted with 10 ml of 10 mM-Tris/HCl buffer, pH 8.4, and applied to a DEAE-Sephacel column (1 cm × 20 cm). The labelled glycosaminoglycans were eluted with 50 ml of a linear gradient of 0–0.6 M-NaCl in 10 mM-Tris/HCl buffer, pH 8.4. Fractions (1 ml) were collected and the radioactivity was determined from samples thereof.

The medium was processed for the analysis of glycosaminoglycans in the following way. Ethanol was

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added to the medium to a final concentration of 80% (v/v), and after 1 h at room temperature the precipitate was sedimented at 2000 *g* for 5 min. The pellet was resuspended in 3 ml of phosphate-buffered saline and digested with 10 mg of Pronase for 24 h at 37 °C. After dilution with 3 ml of 10 mM-Tris/HCl buffer, pH 8.4, the solution was subjected to ion-exchange chromatography as described above. A representative elution profile is shown in Fig. 1. Hyaluronate, heparan sulphate and chondroitin sulphate were eluted at 0.35 M-, 0.435 M- and 0.52 M-NaCl respectively. The radioactivity of the fractions was calculated after subtraction of the background.

RESULTS

Hyaluronate production by synchronized human fibroblasts

Human fibroblasts were grown in the presence of [³H]glucosamine, and glycosaminoglycans were determined in the medium and in the cell layer by ion-exchange chromatography (Fig. 1). Hyaluronate was the major glycosaminoglycan. When the cell cycle was blocked by colchicine, the amount of hyaluronate in the medium was decreased. Cytochalasin stimulated hyaluronate synthesis in the medium about 10-fold and decreased it by about 30% in the cell layer. Apart from an apparent inhibitory effect of cytochalasin on the production of heparan sulphate, the biosynthesis of the sulphated glycosaminoglycans was less affected by drug treatment (Table 1).

The kinetics of hyaluronate synthesis by untreated cultures and cells synchronized by colchicine or cytochalasin were compared. In non-synchronized cells the amount of hyaluronate gradually increased in the cell layer and in the medium (Fig. 2). The control cells had normal morphology. When colchicine was present for 40 h in the culture, little hyaluronate appeared in the medium, and the cell layer contained amounts of hyaluronate comparable with those in non-synchronized cells (Fig. 3). The cells were also well spread on the substratum. When the colchicine block was removed by changing to normal medium, there was an enhanced production of hyaluronate, which appeared first in the cell layer and was subsequently shed into the medium.

Fig. 1. Ion-exchange chromatography of glycosaminoglycans from HE-19 cells labelled with [³H]glucosamine for 40 h

Details are given in the Materials and methods section. ○, Radioactivity; ----, [NaCl].

This was in contrast with the effect of exposure to cytochalasin, where most cells were round and secreted large amounts of hyaluronate into the medium after 25 h (Fig. 4). The amount of hyaluronate at the cell layer remained relatively low. These results indicate that cell rounding during mitosis was accompanied by a marked increase of hyaluronate synthesis.

When the degree of synchrony after release of the cytochalasin block was determined by thymidine incorporation, the cells reached the S-phase after 15 h in normal medium (Fig. 5). A 5-fold increase in [³H]thymidine incorporation into DNA at 15–20 h after drug release indicated a high degree of synchrony and confirmed that prolonged exposure to cytochalasin did not interfere with subsequent DNA replication.

Hyaluronate synthase activity during cytochalasin block

Since the increase in hyaluronate synthesis could be due to an activated hyaluronate synthase or to other metabolic effects such as increased intracellular precursor pools of UDP-glucuronic acid and UDP-*N*-acetylglucosamine, we determined the hyaluronate synthase activity of cells harvested at different times after exposure to

Table 1. Incorporation of [³H]glucosamine into glycosaminoglycans by HE-19 cells

HE-19 cells (5×10^6) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2.5 μ Ci of [³H]glucosamine/ml in the absence and in the presence of cytochalasin (2.0 μ g/ml) or colchicine (0.01 μ l/ml). The radioactivity from the cell layer and the medium were separated by ion-exchange chromatography, and the incorporation into hyaluronate, chondroitin sulphate and heparan sulphate per 10^6 cells was determined as described in the Materials and methods section. Abbreviation: N.D., not detected.

	Incorporation of [³ H]glucosamine (c.p.m./ 10^6 cells)					
	Hyaluronate		Heparan sulphate		Chondroitin sulphate	
	Cells	Medium	Cells	Medium	Cells	Medium
Normal DMEM	1740	4765	2168	N.D.	3071	N.D.
DMEM containing cytochalasin	1112	41900	364	N.D.	2096	N.D.
DMEM containing colchicine	1714	1416	1046	N.D.	3334	N.D.

Fig. 2. Hyaluronate production by non-synchronous human fibroblasts

(a) Human fibroblasts HE-19 (bar represents 50 μm) were labelled with [^3H]glucosamine and (b) the radioactivity incorporated into hyaluronate of the cell layer (■—■, left-hand scale) and into the medium (●—●, right-hand scale) was determined as described in the Materials and methods section. The medium was changed at 40 h (arrow). The amount of radioactivity incorporated into hyaluronate is expressed per 10^6 cells at the time of harvest.

Fig. 3. Hyaluronate production during synchronization of HE-19 cells with colchicine

(a) Colchicine was added at a concentration of 0.01 $\mu\text{g}/\text{ml}$ to HE-19 cells (bar represents 50 μm) and (b) hyaluronate production was determined as indicated in Fig. 2 legend. After 40 h the cells were transferred to normal growth medium (arrow).

cytochalasin. Fig. 6 shows that hyaluronate synthase activity increased during prolonged exposure to cytochalasin and that this increase correlated with hyaluronate production. The initial high synthase activity in the control and the cytochalasin-treated culture was probably due to serum stimulation after subcultivation (Tomida *et al.*, 1977). When the cytochalasin block was released after 40 h by change of medium, the synthase activity rapidly decreased (Fig. 6). At this time even new medium did not stimulate the synthase.

Block of the cell cycle by an inhibitor of hyaluronate synthase

The increase in hyaluronate production during mitosis might either be important or be coincidentally caused by side effects of cytochalasin or colchicine treatment. This ambiguity could be resolved by inhibition of hyaluronate synthesis and studying mitosis in living cells. When periodate-oxidized UDP-glucuronic acid was introduced into cells by hypo-osmotic lysis of pinocytotic vesicles (Okada & Rechsteiner, 1982), cell growth was blocked (results not shown). It was therefore possible that the lack of hyaluronate synthesis during cell detachment in mitosis was responsible for growth inhibition. Therefore

Fig. 4. Hyaluronate production during synchronization of HE-19 cells with cytochalasin

(a) Cytochalasin was added at a concentration of $2.0 \mu\text{g/ml}$ to HE-19 cells (bar represents $50 \mu\text{m}$) and (b) production of hyaluronate was determined as indicated in Fig. 2 legend. After 40 h the cells were transferred to normal growth medium (arrow).

we synchronized human fibroblasts by the use of colchicine or cytochalasin, inhibited hyaluronate synthesis by introduction of periodate-oxidized UDP-glucuronic acid into living cells and determined, by measuring thymidine incorporation, whether or not the cells proceeded to S-phase. The cells were cultivated for 40 h in the presence of colchicine or cytochalasin and then transferred to normal growth medium. This is indicated as zero time in Figs. 7 and 8. Immediately thereafter periodate-oxidized UDP-glucuronic acid was introduced into the cytoplasm, and progress through the cell cycle was determined by labelling the cells with $[^3\text{H}]$ thymidine for 30 min before they were harvested at the times indicated in Figs. 7 and 8.

Fig. 5. Determination of HE-19 cell synchrony

Normal cells (▲) and cells blocked with cytochalasin (●) for 40 h were transferred to normal growth medium at time zero and labelled with $[^3\text{H}]$ thymidine (5 Ci/mmol ; $0.5 \mu\text{Ci/ml}$) for 30 min at the times indicated. The incorporation into DNA per 10^6 cells was determined as described previously (Scott *et al.*, 1956).

Fig. 6. Hyaluronate synthase activity of HE-19 cells during cytochalasin block

Cells were plated in the presence (▲) and in the absence (●) of cytochalasin ($2.0 \mu\text{g/ml}$) at time zero and at the times indicated harvested in phosphate-buffered saline with the aid of a rubber 'policeman'. The cells were centrifuged at $1000 g$ for 5 min and suspended at 10^7 cells/ml in phosphate-buffered saline. The suspension was frozen and thawed once to disrupt the membranes, and the hyaluronate synthase activity was determined as described previously (Prehm, 1980). The cytochalasin block was released after 40 h.

Fig. 7. Thymidine incorporation by HE-19 cells synchronized by cytochalasin

HE-19 fibroblasts were blocked with cytochalasin for 40 h. Then the cells were transferred to normal growth medium without the drug and divided into two parts. This is taken as time zero. One sample was treated with periodate-oxidized UDP-glucuronic acid (●) as described previously (Prehm, 1985) and the other part served as a control (▲). Synchrony was determined as indicated in Fig. 5 legend.

Fig. 8. Thymidine incorporation by HE-19 cells synchronized by colchicine

HE-19 fibroblasts were blocked with colchicine for 40 h. Then the cells were transferred to normal growth medium without the drug and divided into two parts. This is taken as time zero. One sample was treated with periodate-oxidized UDP-glucuronic acid (●) as described previously (Prehm, 1985) and the other part served as a control (▲). Synchrony was determined as indicated in Fig. 5 legend.

When periodate-oxidized UDP-glucuronic acid was introduced after the cytochalasin block, the cells entered the S-phase at the same time as did control cultures that had been exposed to the pinocytotic medium lacking

periodate-oxidized UDP-glucuronic acid (Fig. 7). However, when hyaluronate synthase was inhibited after the colchicine treatment, but before the rounding of cells in mitosis, the cells remained firmly attached to the substratum and did not reach the S-phase (Fig. 8).

When the cells were subjected to the treatment with colchicine or cytochalasin without subsequent exposure to periodate-oxidized UDP-glucuronic acid (Figs. 7 and 8), they reached the S-phase at the same time as was observed before (Fig. 4), confirming previous observations (Okada & Rechsteiner, 1982; Prehm, 1985) that hypo-osmotic lysis of pinocytotic vesicles was not toxic to cells.

This experiment demonstrated that inhibition of glycosaminoglycan synthesis arrested the cell cycle before mitotic detachment.

DISCUSSION

The drugs colchicine (Stubblefield *et al.*, 1967) and cytochalasin (Carter, 1967) arrest the cell cycle in the prophase of mitosis and in cytokinesis respectively. Colchicine inhibits spindle formation by interfering with microtubule formation, and cytochalasin blocks actin filament formation and cell division. Cells in pro-phase are attached to the substratum, but they round up in cytokinesis and are loosely adherent. Dramatic changes on the cell surface and in the extracellular matrix accompany the mitotic process during the 30 min between pro-phase and cytokinesis. Proteinases are believed to be responsible for detachment and rounding of cells (Hatcher *et al.*, 1976). Shedding of cell-surface heparan sulphate proteoglycan has been shown to precede mitosis (Kraemer & Tobey, 1972). Otto & Mühlradt (1980) observed that glycosaminoglycans were preferentially synthesized and secreted after the S-phase.

We observed that human fibroblasts synchronized either with colchicine or with cytochalasin showed an increased hyaluronate synthesis at the time of cell rounding in mitosis. The increase in hyaluronate synthesis was maintained during the cytochalasin block in cytokinesis, but declined sharply as the cells entered the G₁-phase and resumed spread morphology. The synthesis of other glycosaminoglycans was less affected by this treatment and low compared with that of hyaluronate.

The inhibition of the synthesis of other glycosaminoglycans had been determined previously with HT-1080 cells (Prehm, 1985). These experiments showed that periodate-oxidized UDP-glucuronic acid was not specific, because it similarly interfered with the synthesis of chondroitin sulphate and heparan sulphate. Thus it might also inhibit mitosis by blocking the synthesis of other components. However, combining the observations that mitosis was accompanied by elevated hyaluronate synthesis and unchanged chondroitin sulphate and heparan sulphate synthesis and that mitosis was blocked by inhibition of glycosaminoglycan synthesis suggested that hyaluronate synthesis was required for mitotic detachment.

There are many indications that hyaluronate interferes with the interaction of matrix components and cell attachment. Hyaluronate inhibits the heparan sulphate-stimulated aggregation of fibronectin and collagen (Hörmann & Jelinic, 1981). Cell variants with high substratum adhesiveness produce less (Kraemer &

Barnhart, 1978) whereas variants with lower adhesiveness produce more hyaluronate (Barnhart *et al.*, 1979). Hyaluronate-containing matrix is a poor attachment substratum for cell growth (Fisher & Solursh, 1979), and exogenous hyaluronate facilitates detachment (Abatangelo *et al.*, 1982). Hyaluronate synthesis has also been correlated with cell migration (Toole, 1972) and tumour-cell invasion (Toole *et al.*, 1979). Analysis of glycosaminoglycans in substratum-adhesion sites of migrating cells suggested that hyaluronate secretion labilized fibronectin-mediated attachment (Culp, 1978).

Hyaluronate could serve similar functions in all these cellular processes, namely disruption of adhesive plaques or cell contacts. A basal level of hyaluronate synthesis during the G₁-, S- and G₂-phases could be used for cell migration, if it were synthesized at the trailing edge of a migrating cell. During mitosis the synthase activity could be activated at all cellular contact areas and result in cell detachment and rounding.

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