Time-Course Experiment Involving Glycosaminoglycan Synthesis by Normal Human Dermal Fibroblasts Cultured in the Presence of Dibutyryl Cyclic AMP and Retinoic Acid

Toshio Imanari,*a Makoto Saito,5 Guoning Qiu,* Toshihiko Toida,* and Hiroshi Akiyama*

Faculty of Pharmaceutical Sciences, Chiba University,* 1–33 Yayoi-cho, Inage-ku, Chibashi, Chiba 263, Japan and Noda Institute of Scientific Research,* 399 Noda, Noda-cho, Chiba 278, Japan.

Received March 30, 1994; accepted August 25, 1994

Glycosaminoglycan (GAG) synthesis by cultured normal human dermal fibroblasts was examined. Hyaluronic acid (HA) synthesis reached a maximum on day 3 (0.3 μg/ml medium) and then decreased to a low level (0.15 μg/ml medium). The amounts of dermatan sulfate (DS) and chondroitin sulfate (CS) synthesized by the cells increased with increasing cell numbers during the initial stage to attain constant levels (0.092 μg DS/ml, 0.026 μg CS/ml) after the cells reached confluence. We also tested the effects of dibutyryl cyclic AMP (dbcAMP) and retinoic acid (RA) on GAG synthesis by the cells. The synthesis of HA and CS by cells was stimulated, when the cells were cultured in medium containing dbcAMP (0.1 mM), whereas DS synthesis was scarcely affected. However, addition of RA (5 mM) suppressed GAG synthesis by the cells.

Keywords glycosaminoglycan; fibroblast; dibutyryl cyclic AMP; retinoic acid; skin

Skin tissue consists of epidermal keratinocytes, dermal fibroblasts and extracellular matrix (ECM) composed of compounds such as collagen, glycosaminoglycans (GAGs) and fibronectin. Skin GAGs included hyaluronic acid (HA), chondroitin sulfate (ChS) and dermatan sulfate (DS),1,2 and these play important roles in skin function. In addition, dibutyryl cyclic AMP (dbcAMP)3 and retinoic acid (RA)4−6) are known to affect the growth and differentiation of skin cells. Therefore, the effect of these substrates on GAG synthesis by skin cells is very interesting.

In previous work, we established a new method for the micro-determination of GAGs and used it to monitor GAG synthesis in cultured human epidermal keratinocytes.7 By means of general observations on HA synthesis together with examination of the morphological changes and keratinization of the cells, we succeeded in measuring the effects of RA and dbcAMP on the biosynthesis of HA by cells.

In this paper, we have attempted to analyze GAG synthesis by cultured normal human dermal fibroblasts, and investigated the effect of RA and dbcAMP on GAG biosynthesis.

MATERIALS AND METHODS

Materials A FibroPack kit [including normal human dermal fibroblasts (NHDF) and fibroblast growth medium (F-GM)] was purchased from Sanko Junyaku Co. (Japan); Tissue culture plates, Falcon 3046 (9.6 cm²/well × 6), and sterile plastic pipettes were obtained from Becton Dickinson Co. (UK), and Sterivex-GS sterile filter units were obtained from Millipore Co. (U.S.A.); The CO₂ incubator IT42 was obtained from Yamato Scientific Co. (Japan); Unsaturated disaccharides [2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-glucose (ΔDi-HA), 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-glucose (ΔDi-6S), 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfo-D-galactose (ΔD-4S), 2-acetamido-2-deoxy-3-O-(2-O-sulfogluco-4-epi-pyranosyluronic acid)-4-O-sulfo-D-galactose (ΔD-diS)] and chondroitinase ABC (ChaseABC, EC 4.2.2.4) and chondroitinase ACII arthro (ChaseACII, EC 4.2.2.5) were purchased from Seikagaku Kogyo Co. (Japan); TSKgel NH₂-60 for the HPLC column packing was obtained from Tosoh Co. (Japan). All other chemicals were of analytical grade. Reagent solutions were made up freshly in water prepared by de-ionization and distillation.

Culture of Normal Human Dermal Fibroblasts Sub-confluent cells (2nd passage) were washed twice with N-2-hydroxyethylpipperazine-N'-2-ethanesulfonic acid (HEPES) buffer solution (pH 7.4) at 37 °C, and treated with 0.025% trypsin/0.01% EDTA solution for 1−2 s. The cells were then washed with a trypsin-neutralizing solution and suspended in fresh F-GM medium (Sanko Junyaku Co. Japan). After the suspension was centrifuged at 220 × g for 10 min at room temperature, the fibroblasts were resuspended in fresh medium and cell numbers were counted using a hemocytometer. The cells were inoculated in plastic culture dishes (9.6 cm²) at an initial density of about 5 × 10³ cells/dish and cultured with 2 ml F-GM at 37 °C in a humidifier incubator in an atmosphere of 5% CO₂, 95% air. The medium was replaced every two days and the old medium was stored at −20 °C until being analyzed.

Addition of dbcAMP and RA to Fibroblast Culture Medium For the addition of dbcAMP,200 μl dbcAMP solution (1.0 mM in physiological saline) was mixed with 1.8 ml fresh medium for replacement. For the addition of RA, 10 μl RA solution (1.0 mM in ethanol) was mixed with 190 μl 4% albumin and left to stand for 1 h at 37 °C in the dark with shaking, then mixed with fresh medium (1.8 ml) prepared for replacement. The additions of RA (5 mM) and dbcAMP (0.1 mM) to culture medium were made at the sub-confluent stage (day 7). Cell culture
medium containing the chemicals was continuously replaced once every two days during cell culture.

**Determination of HA, ChS and DS in Culture Medium**

The preparation method of the determination for GAGs was carried out as described previously. A 200 µl volume of culture medium was transferred to a microtube and heated at 100 °C for 5 min then cooled in a water bath. To this solution was added 50 µl 0.05 M Tris-HCl buffer (pH 8.0) containing 1% Actinase E, and this mixture was incubated at 45 °C for 3 h. Afterwards, cold ethanol saturated with sodium acetate (1 ml) was added, and the mixture was kept overnight at 0 °C, then centrifuged at 2300 × g for 15 min. The precipitate was washed twice with 500 µl 80% ethanol and dried in vacuo. The residue was dissolved in 25 µl 0.1 M Tris-HCl buffer (pH 8.0) containing both ChaseABC and ChaseACII (0.025 U each) or in 25 µl 0.1 M acetate buffer (pH 6.0) containing only ChaseACII (0.05 U) and left to incubate at 37 °C for 3 h. Four volumes of ethanol (saturated with sodium acetate) was added to the solution and mixed well. Finally, 20 µl of supernatant obtained by centrifugation at 2300 × g for 15 min was directly submitted to HPLC to determine the disaccharide composition.

**RESULTS**

The time-courses of the glycosaminoglycan (GAG) levels in fibroblast medium were examined and the results are shown in Fig. 1. During the experiment, fibroblasts were cultured for 17 d and the newly synthesized GAGs in the medium were determined. Another parallel control experiment was carried out to count the cell numbers as described in the experimental section. From these results, it was found that the major GAG components synthesized by the cells were HA and DS, together with small amounts of ChS. The activation of HA synthesis occurred during the initial stage of the cell growth and HA synthesis by the cells reached a maximum on day 3 (0.3 ± 0.04 µg/ml medium), then rapidly fell to a constant low level (0.15 ± 0.02 µg/ml medium), after the cells reached confluence. This result was consistent with that obtained with cultured mouse skin fibroblasts.

In contrast to the HA synthesis, the DS and ChS concentrations in culture medium increased with increasing cell numbers during the initial stage, to attain constant levels (0.092 ± 0.03 µg DS/ml, 0.026 ± 0.02 µg ChS/ml medium) after the cells reached confluence. These observations may reflect the constant capacity per cell of the fibroblasts with respect to DS and ChS synthesis during cell culture.

Next, we studied the effects of dbcAMP and RA on cultured fibroblasts maintaining the cultures for 30 d.

Figure 2 shows the effects of dbcAMP on GAG synthesis by cells. It can be seen that dbcAMP stimulates HA synthesis markedly during cell culture. In particular, the HA synthesis by the cells on day 11 (4.8 ± 1.0 µg/ml medium) was about thirty-fold higher than that of the control cultures (0.16 ± 0.03 µg/ml medium).

Although DS synthesis by the cells was hardly affected by the addition of dbcAMP, ChS synthesis was stimulated (10-fold for ΔDi-6S, 2-fold for ΔDi-4S). In addition, we...
FIG. 3. Effects of RA on GAG Synthesis in Normal Human Dermal Fibroblasts in Culture

Retinoic acid was added to the culture medium (final concentration, 5 nM) when the cells became sub-confluent (day 6, shown by arrow). Each point represents the mean ± S.E. of three experiments. In the control culture, F-GM containing 4% of bovine serum albumin was used. —○—, control; —●—, RA; ——, returned to control culture conditions.

also observed GAG synthesis by the cells on stopping the addition of dbcAMP after the cells were cultured for 14 d. However, GAG synthesis returned to the same level as the control when the addition of dbcAMP to the culture medium was stopped.

Figure 3 shows the effects of RA on GAG synthesis by fibroblasts. These results show that the capacity for HA and DS synthesis by the cells decreased gradually due to the addition of RA, while ChS increased slightly 2–4 d after addition of RA and then decreased gradually. The effect of RA on GAG synthesis was reversible because GAG synthesis returned to control levels when the addition of RA was stopped.

DISCUSSION

In previous work,7 we developed a new method for monitoring GAG synthesis by cells in culture medium. By means of our method, trace amounts of GAGs synthesized by cultured cells can be determined over a longer period than by traditional methods which used radioactive materials such as $^{35}$SO$_4^{2-}$.10

HA synthesis in cultured fibroblasts showed a transient increase in early culture in contrast to sulfated GAGs. It has been reported that GAG synthesis is regulated by cell size and the extent of cell–cell contact11 and proteoglycans (PG) have been implicated in numerous processes such as regulation of cell adhesion and control of cell growth.12,13 It has been also shown that betaglycan, which is a cell surface PG containing both heparan sulfate (HS) and ChS chains, binds basic fibroblast growth factor through its HS chain.14 Therefore, the initial increase in HA may be related to the promotion of normal human dermal fibroblast growth by modifying the action of growth factors.

Additionally, it has been reported that dbcAMP13 and RA4–6 are associated with alterations in the differentiation and growth of dermal cells. In this work, we found that the synthesis of GAG was markedly affected by the presence of dbcAMP or RA in the culture medium. The regulatory mechanisms by these compounds, which were used to control GAG biosynthesis, are still not clear. However, these compounds may regulate gene transcription of the enzyme groups responsible for GAG synthesis through so-called signal transduction systems in a similar manner to calcium, which regulates the synthesis of heparan sulfate PG in breast cancer cells.15 Consequently, more investigations are required to examine the relationship between GAG biosynthesis and these compounds.

REFERENCES