Analytical Studies on Hyaluronic Acid Synthesis by Normal Human Epidermal Keratinocytes Cultured in a Serum-Free Medium

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Hyaluronic acid (HA) synthesized by normal human epidermal keratinocytes cultured in a serum-free medium was monitored by a highly sensitive HPLC method, which was established by us for the simultaneous determination of HA, chondroitin sulfate (Cs) and dermatan sulfate (DS) as their unsaturated disaccharides. The major glycosaminoglycan (GAG) in the medium of the keratinocytes was HA, and the ability of the cells to synthesize HA increased relatively with an increase in cell numbers during the logarithmic phase and reached a maximum level after the cells became confluent. HA synthesis by the keratinocytes was inhibited by the addition of calcium chloride to the culture medium, and was strongly stimulated by the addition of retinoic acid (RA), respectively. It was shown that the ability of the cells to synthesize HA exists in the spinous cell stage. Furthermore, we found that HA synthesis by the cells was slightly increased by the addition of dibutyl cyclic AMP (dbcAMP). Our results indicate that the measurement of time-course levels of HA in the culture medium is useful for the screening of active substances for proliferation and differentiation of the keratinocytes.

Keywords hyaluronic acid; keratinocytes; calcium ion; retinoic acid; dibutyl cyclic AMP

Hyaluronic acid (HA) is one of major glycosaminoglycans (GAGs) in the extracellular matrices (ECM) of mammalian epidermal keratinocytes, and its synthesis and degradation are significantly related to cellular functions.1) The ability of keratinocytes to synthesize HA has been used as the biological model to study HA both in cell and organ cultures.2)−12) However, in most of these studies, radioactive substances were used to detect and to determine the synthesis of HA, but these substances sometimes affect cell growth or the synthesis of the ECM of the cells.

On the other hand, the effects of calcium ion,13) retinoic acid (RA)14) and dibutyl cyclic AMP (dbcAMP)15) in the culture of keratinocytes have been widely studied, and it is clear that they affected cell growth and differentiation through different biological pathways.

In this paper, we established an accurate and highly sensitive system for the analysis of HA synthesized by normal human epidermal keratinocytes, and the levels of HA synthesized were then examined after the addition of calcium chloride, RA or dbcAMP to the medium, in order to understand the relationship between cell-growth and the ability of the cells to synthesize HA.

MATERIALS AND METHODS

Reagents and Materials An EpiPack kit [including keratinocytes (NHEK) and a culture medium (K-GM)] was purchased from Seikagaku Kogyo Co. (Japan). Tissue culture plates, Falcon 3046 (9.6 cm/well × 6), and sterile plastic pipettes were from Becton Dickinson Co. (UK), and a Sterivex-GS sterile filter unit was obtained from Millipore Co. (U.S.A.). A CO2 incubator IT42 was from Yamato Scientific Co. (Japan), unsaturated disaccharide [2-acetamido-2-deoxy-3-O-(β-D-glucos-4-eneprynosyl-uronic acid)-D-glucose (ΔDi-HA)], chondroitinase ABC (EC 4.2.2.4) and chondroitinase ACII arthro (EC 4.2.2.5) were purchased from Seikagaku Kogyo Co. (Japan). Actinase E was from Kaken Pharmaceutical Co. (Japan). TSKgel NH2-60 for a 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 deionization and distillation.

Culture of Normal Human Epidermal Keratinocytes Sub-confluent cells were washed with a 0.025% trypsin/0.01% EDTA solution for 30−60 s, and by a trypsin neutralizing solution. They were then suspended in the fresh medium. After centrifugation at 220 × g for 10 min at room temperature, the keratinocytes were resuspended in the fresh medium, and cell numbers were counted by a hemocytometer. The cells were inoculated in plastic culture dishes (9.6 cm2) at an initial density of about 2 × 105 cells/dish and cultured with 2 ml K-GM at 37°C in a humidifier incubation under 5% CO2, 95% air. The medium was replaced every two days and stored at −20°C until being used. In the case of adding active substances, the media which contained calcium chloride (1 mM), RA (5 μM) or dbcAMP (0.1 mM) were replaced every two days after the cells became sub-confluent.

Cells obtained in the culture conditions described above were treated with 200 μl of 50 mM Tris–HCl buffer containing 0.1% sodium dodecyl sulfate (SDS) and 0.1% mercaptoethanol for 30 min, then their insoluble cornified envelopes were counted by a hemocytometer.

Determination Method of HA in Culture Medium In order to determine the GAGs in the medium, 200 μl of the medium was transferred to a microtube, then heated in a boiling water bath for 5 min. After cooling the mixture in an ice bath, it was added sequentially with 50 μl actinase E [1% in 50 mM Tris–HCl buffer (pH 8.0)] then incubated at 45°C for 3 h. After the addition of 1 ml of cold ethanol saturated with sodium acetate, the mixture was kept overnight at 0°C. Next, the precipitate obtained from centrifugation at 2300 × g for 15 min was washed twice
with 500 μl of 80% ethanol, then dried *in vacuo*. The residue was dissolved in 25 μl of 0.1 M Tris–HCl buffer (pH 8.0) containing both chondroitinase ABC and ACII (0.025 unit each), and the mixture was incubated at 37°C for 3 h. To the mixture, 100 μl of cold ethanol saturated with sodium acetate was added, and the new mixture was kept overnight at 0°C. After being centrifuged at 2300 × g for 15 min, aliquots of the supernatant were successively submitted to the HPLC system for quantitative analysis of HA. The extraction efficiency of standard HA from the medium under this pre-treatment procedure was about 95% (> 0.1 μg HA/ml K-GM, n = 12).

**Apparatus**  The determination of unsaturated disaccharides of HA, chondroitin sulfate (ChS) and dermamin sulfate (DS) was carried out using the HPLC systems described previously, and the conditions were as follows: A 10–20 μl portion of sample solution was loaded *via* a sample injector with a 100 μl loop. A TSKgel NH2-60 column (4.6 mm i.d. × 250 mm) was eluted with 40 mm Tris–HCl borate buffer (pH 7.5 adjusted with HCl) and 5 mm sodium sulfate in 54% acetonitrile at a flow-rate of 0.5 ml/min. To the eluate were added a 0.3 M sodium hydroxide solution and aqueous 1% 2-cyanoacetamide solution at a flow-rate of 0.25 ml/min controlled by a double plunger pump. The mixture was then passed through a polytetrafluoroethylene (PTFE) reaction coil (0.5 mm i.d. × 10 m) set in a dry reaction bath thermostated at 105°C, followed by a PTFE cooling coil (0.25 mm i.d. × 2 m) in a water bath at 25°C. The effluent was monitored by a fluoromonitor (Ex. 346 nm, Em. 410 nm).

**RESULTS AND DISCUSSION**

HA levels in the medium cultured with normal human epidermal keratinocytes for 16 d were measured. To observe the cell growth and differentiation, another parallel control experiment was carried out to count the cell numbers and cornified envelopes (an indicator of the keratinization of keratinocytes) according to the methods described in the experimental section. These results are shown in Fig. 1. The cultured keratinocytes reached a confluent stage on day 8 after inoculation. The amount of cornified envelopes increased gradually from day 6 to day 12; the concentration of HA in the culture medium increased rapidly, together with increasing cell numbers during the logarithmic phase, and reached a maximum level on day 10 (0.8 μg HA/ml medium), then decreased immediately following the keratinization of the cells.

Next, the following experiments were designed to test the effects of active substances on the synthesis of GAGs in culture. The calcium ion is known to activate the keratinization of keratinocytes. The addition of calcium chloride (1 mM) was started at the sub-confluent stage (day 6). Distinctive morphological changes in the cells were recognized by microscopic observation after the addition of calcium chloride. The keratinocytes seemed to be tightly connected to each other, with increased desmosomes. As shown in Fig. 2B, the cornified envelope formations of the cells after the addition of calcium chloride were about two times higher than that of the control (which also contained small amounts of Ca2+, 0.03 mM). These results indicate that the addition of a calcium chloride to a culture medium stimulates the terminal differentiation of the keratinocytes in culture. HA freshly synthesized in the medium was also analyzed and the results are shown in Fig. 2A. The HA level decreased due to the addition of calcium chloride. This phenomenon suggests that the capability for HA synthesis by keratinocytes decreases with the keratiniza-

![Fig. 1. Time-Courses of HA Levels, Cell Numbers and Cornified Envelopes during the Culture of Normal Human Epidermal Keratinocytes](image)

Each point represents the mean ± S.E. of three experiments.

![Fig. 2. Influence of Calcium Ion on HA Synthesis by Normal Human Epidermal Keratinocytes](image)

- O --- control; - • --- calcium ion. A: Calcium chloride was added to the culture medium (final concentration, 1 mM) 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, K-GM (containing 0.03 mM of Ca2+) was used. B: Histograms showed the cornified envelopes on day 14.
Fig. 3. Influence of Retinoic Acid (RA) on HA Synthesis by Normal Human Epidermal Keratinocytes

—○—, control; —●—, retinoic acid. A: Retinoic acid was added to the culture medium (final concentration, 5 μM) 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, K-GM containing 4% of bovine serum albumin was used. B: Histograms showed the cornified envelopes on day 14.

Fig. 4. Influence of Dibutyl cyclic AMP (dbcAMP) on HA Synthesis by Normal Human Epidermal Keratinocytes

—○—, control; —●—, dbcAMP. A: dbcAMP was added to the culture medium (final concentration, 0.1 mM) 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, K-GM was used. B: Histograms showed the cornified envelopes on day 14.

tion of the cells.

It is reported that RA suppressed the keratinization of keratinocytes,\(^ {14}\) in contrast to the effect of the calcium ion. The cornified envelope formation of cultured keratinocytes in the present experiment on day 14 was about half of the control (Fig. 3B). In the microscopic observation of the cells, two days after the addition of RA, remarkable changes in the morphology of the cells were observed compared with that of the control. Contrary to the case in which calcium chloride was added, the formation of desmosome was also suppressed (data not shown). HA synthesis in the medium increased remarkably after the addition of RA (Fig. 3A). HA synthesis by keratinocytes has been thought so far to take place primarily during the spinous cell phase, which is one of the differentiation stages of the cells.\(^ {15}\) It is estimated that spinous cells stayed longer to synthesize HA, because RA suppressed the keratinization of the cells.

Cyclic AMP (cAMP) and its analogs were widely investigated as substances which induce the differentiation of several mammalian cell lines.\(^ {17}\) Figure 4A depicts changes in HA synthesis and in the keratinization of the keratinocytes by the addition of dbcAMP (0.1 mM) in a culture medium. The cells tightly connected to each other, and desmosomes increased. This morphological effect was similar to that caused by the addition of calcium ion. The cornified envelopes after the addition of dbcAMP on day 14 increased about 3 times more than that of the control (Fig. 4B). However, the capability for HA synthesis by the keratinocytes in a culture medium still increased slightly. This result was in conflict with the above conclusion obtained from the addition of calcium chloride or RA. It is suggested that dbcAMP may significantly stimulate HA synthesis of the cells during the spinous phase. Furthermore, the mechanisms of stimulation by dbcAMP on HA synthesis are not clear; therefore, more studies are required to understand the biochemical significance of cAMP.

As described above, we demonstrated a method to determine the considerably low concentration of HA synthesized by culture keratinocytes in the medium by the use of a sensitive HPLC method. This method could monitor the time-course levels of HA during culture, so is applicable for research on active substances for the proliferation and differentiation of keratinocytes in culture.

REFERENCES