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EXTRACELLULAR DEPOLYMERIZATION OF HYALURONIC ACID IN CULTURED HUMAN SKIN FIBROBLASTS[1]

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Summary: The chain length of [^{3}H]hyaluronic acid synthesized by cultivating human skin fibroblasts in the presence of [^{3}H]glucosamine was investigated. [^{3}H]Hyaluronic acid obtained from the matrix fraction was excluded from a Sepharose CL-2B column irrespective of the incubation period, whereas that from the medium was depolymerized into a constant chain length (Mr=40,000). The reducing and non-reducing terminals of the depolymerized hyaluronic acid were N-acetylglucosamine and glucuronic acid, respectively. Prolonged incubation produced no oligosaccharides as shown by examination of hyaluronidase digests, suggesting the presence of a novel endo-β-N-acetylglucosaminidase in cultured human skin fibroblasts.

Hyaluronic acid is widely distributed in tissue as one of the major components of the extracellular matrix. Its catabolism seems to occur through cleavage of the internal bonds of the sugar chain by hyaluronidase (endo-β-N-acetylhexosaminidase), the resulting oligosaccharides then being degraded by exo-β-glucuronidase and exo-β-N-acetylhexosaminidase stepwise from the non-reducing terminal (1).

The degradation process of hyaluronic acid in skin has not been established. It is evident that hyaluronidase is present in rat skin (2) and cultured chick embryonal skin fibroblasts (3). On the other hand, Arbogast et al. (4) have demonstrated an absence of hyaluronidase in cultured human skin fibroblasts. Since then, no studies have succeeded in demonstrating the

[1] All sugars mentioned in this paper have D configurations.

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Abbreviation used; CMF-PBS, calcium- and magnesium-free phosphate-buffered saline.

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presence of hyaluronidase activity in cultures of this cell type. In addition, no internalization of hyaluronic acid by cultured human skin fibroblasts has been observed (5). Therefore, the mechanism of hyaluronic acid degradation in human skin fibroblasts is not yet understood. In the present study, in order to help resolve this issue, changes in the chain length of hyaluronic acid during fibroblast incubation were investigated. It was found that hyaluronic acid in the matrix fraction was of high molecular weight, whereas that shed into the medium was depolymerized with increased incubation time.

This paper describes the presence of a novel endo-β-N-acetylglucosaminidase, different from "hyaluronidase", in cultured human skin fibroblasts.

MATERIALS AND METHODS

Pulse-chase Experiment: Human skin fibroblasts were cultivated in Eagle's minimum essential medium supplemented with 10% fetal bovine serum plus antibiotics as described previously (6). The cells at confluency in a 100-mm plastic dish (Nunc, Roskilde, Denmark) were incubated in the medium containing 2 μCi/ml [^{3}H]glucosamine (specific activity 40 Ci/mmol, ICN Radiochemicals, Irvine, CA). After 24 h, the medium was removed and the cell layer was washed three times with Dulbecco's calcium- and magnesium-free phosphate buffered saline (CMF-PBS), and then incubation was continued with non-isotopic medium for various periods. At the end of the incubation periods, the each medium was pooled, and the cell layer was washed three times with Dulbecco's CMF-PBS. Cell-washing solutions were combined with the media and used as medium fractions. To the cell layer was added 0.25% trypsin/Dulbecco's CMF-PBS. After incubation at 37°C for 15 min, the lysate was centrifuged. The supernatant (trypsinate) was recovered and used as matrix fractions.

Isolation of Hyaluronic Acid: Isolation of [^{3}H]hyaluronic acid was performed as follows. The matrix and medium fractions were passed individually through a column (1.6 x 38 cm) of Sephadex G-15 (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 7 M urea/50 mM Tris-HCl buffer, pH 7.3. The excluded fractions were applied to a column (1.0 x 15 cm) of DEAE-Sephacel (Parmacia) equilibrated with the same buffer. The adsorbed fractions were eluted by a linear gradient elution system with 0-1.0 M NaCl in the same buffer. The fractions, which were sensitive to digestion with *Streptomyces* hyaluronidase (Seikagaku Kogyo, Tokyo, Japan), were recovered, and then applied to a column (1.0 x 72 cm) of Sepharose CL-2B (Pharmacia) equilibrated and eluted with 1.0 M NaCl.

In some cases, non-isotopic hyaluronic acid was isolated from 1.5 l of pooled medium by the same procedures as described above.

Analysis of Low-molecular-weight Hyaluronic Acid: The low-molecular-weight [^{3}H]hyaluronic acid derived from the medium was isolated by Sepharose CL-2B chromatography as described above, and its molecular weight was estimated by high-performance gel-permeation chromatography using a column (0.8 x 30 cm) of Shodex OHpak KB-803 (Showa Denko, Tokyo, Japan) as described previously (7). The non-reducing terminal sugar of the low-molecular-weight [^{3}H]hyaluronic acid was identified by gel filtration on a column (1.0 x 107 cm) of Sephadex G-25 (Pharmacia) after incubation of the sugar chain with β-N-acetylglucosaminidase (bovine epididymis, Sigma, St. Louis., MO) or a combination of β-glucuronidase (purified from rabbit liver (8)) followed by β-N-acetylglucosaminidase digestion. The reducing terminal of the sugar chain was identified by paper chromatography after reduction of the sugar chain with NaB[^{3}H]$_4$ followed by acid hydrolysis, as described previously (9).

Enzyme Assay: The cells cultured in non-isotopic medium were obtained by trypsin treatment, and then sonicated in 10 mM sodium phosphate buffer, pH 7.3, followed by centrifugation. The supernatant (20 mg protein/ml) was used as an enzyme solution. The activities of hyaluronidase and β-glucuronidase acting on p-nitrophenyl-β-D-glucuronide were determined by the method described previously (8). The activity of β-glucuronidase acting only on non-sulfated glycosaminoglycans was determined as described previously (8). As the enzyme activity was totally inhibited in the presence of 100 mM ammonium sulfate (8), the activity of the enzyme was calculated by subtracting the amount of free glucuronic acid after incubation without ammonium sulfate from that with ammonium sulfate. The activity of β-glucuronidase acting on N-acetylhyalobiuronic acid was determined as described previously (10). The activity of β-N-acetylglucosaminidase was determined by the method of Tarentino and Maley (11).

RESULTS

The enzyme activities related to degradation of hyaluronic acid were measured. As shown in Table 1, activities of exoglycosidases toward p-nitrophenyl derivatives and chondroitin were detected, but no hyaluronidase activity was evident. Furthermore, no N-acetylhyalobiuronic acid-degrading β-glucuronidase activity (10) was detected in cultured human skin fibroblasts.

Changes in the chain length of matrix- and medium-derived hyaluronic acid during incubation were then investigated. The matrix and medium fractions after various incubation periods were applied to a DEAE-Sephacel column. It was found that the amount of ^{3}H-labeled macromolecules in the matrix fraction was decreased during incubation, whereas no such change was evident in the medium fraction (Fig. 1). The fractions found to contain hyaluronic acid on the basis of Streptomyces hyaluronidase digestion (indicated by horizontal

Table 1. Enzyme activities related to degradation of hyaluronic acid in cultured human skin fibroblasts

Enzyme	Substrate	Activity[a]
Hyaluronidase	Hyaluronic acid	N.D.[b]
β-Glucuronidase	p-Nitrophenyl-β-D-glucuronide	13.7
β-Glucuronidase[c]	Chondroitin	1.2
β-Glucuronidase[d]	N-Acetylhyalobiuronic acid	N.D.[b]
β-N-Acetylglucosaminidase	p-Nitrophenyl-β-D-N-acetylglucosaminide	290.0

a. One unit of the enzyme is defined as that liberating 1 μmol of p-nitrophenol or glucuronic acid per min. Values are expressed as mU/mg protein.
b. Not detected.
c. The enzyme is active only on non-sulfated glycosaminoglycans and is different from that acting on p-nitrophenyl-β-D-glucuronide (8).
d. The enzyme is different from that acting on p-nitrophenyl-β-D-glucuronide (10).

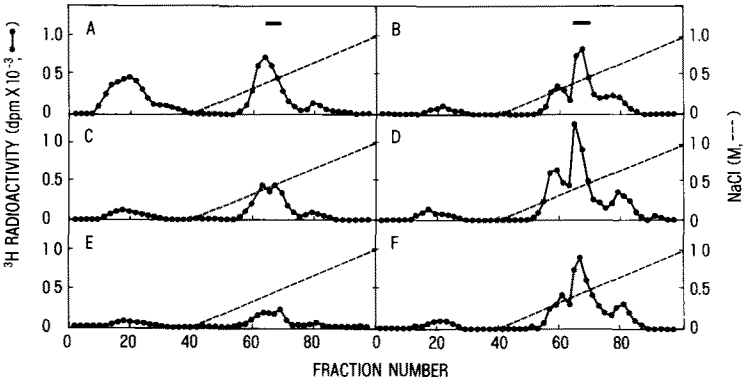

Fig. 1. DEAE-Sephacel chromatograms of ^{3}H-labeled macromolecules derived from matrix (A, C and E) and medium (B, D and F) fractions. A and B, chase-day 1; C and D, chase-day 4; E and F, chase-day 10.

bars in Fig. 1), were collected and applied to a Sepharose CL-2B column. In matrix-derived hyaluronic acid fractions, a minor peak eluted at Vo and a major peak at Vt were always present (Fig. 2 A, C and E). The former was sensitive to Streptomyces hyaluronidase digestion, but the latter was resistant to the enzyme, indicating that the latter was contaminating glycoprotein derived from fractionation by DEAE-Sephacel chromatography (Fig. 1). In the medium fraction, on the other hand, high-molecular-weight hyaluronic acid eluted at Vo was present on chase-day 1, although another accompanying major peak was also observed (Fig. 2 B). The excluded peak, however, disappeared on chase-

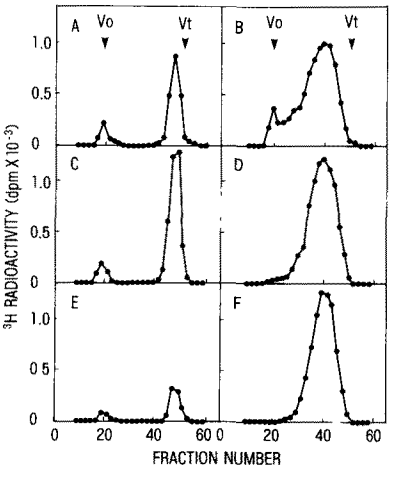

Fig. 2. Sepharose CL-2B chromatograms of [^{3}H]hyaluronic acid fractions derived from matrix (A, C and E) and medium (B, D and F) fractions. A and B, chase-day 1; C and D, chase-day 4; E and F, chase-day 10. Vo, void volume; Vt, total volume.

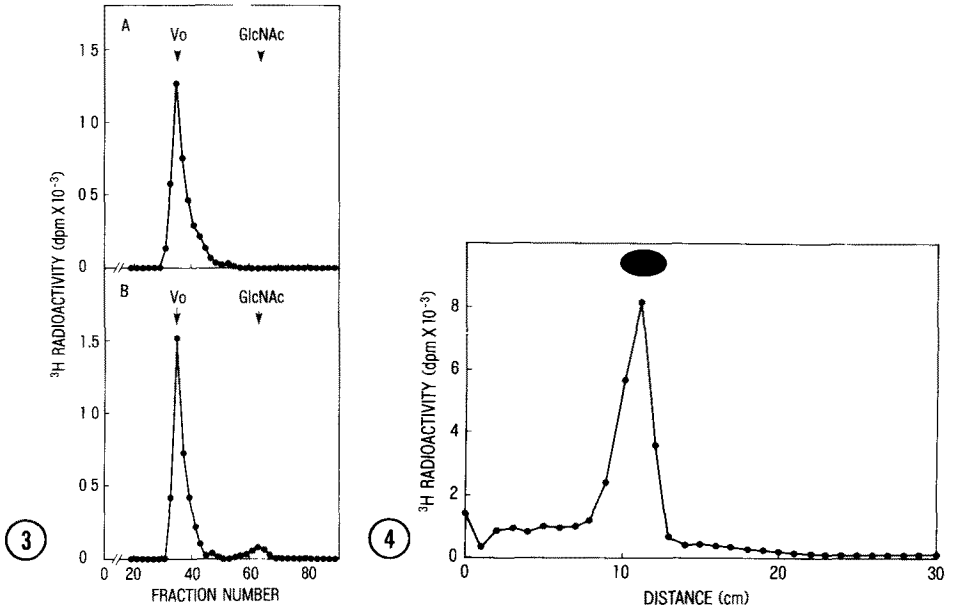

Fig. 3. Sephadex G-25 chromatograms of depolymerized [^{3}H]hyaluronic acid after exoglycosidase treatment. A, after incubation with β-N-acetylglucosaminidase; B, after incubation with β-glucuronidase followed by incubation with β-N-acetylglucosaminidase. Vo, void volume; GlcNAc, elution position of N-acetylglucosamine used as a standard.

Fig. 4. Paper chromatograms of ^{3}H-labeled alditol at the reducing terminal of depolymerized hyaluronic acid. Depolymerized hyaluronic acid was reduced with NaB[^{3}H]$_4$, followed by hydrolysis with 4 N HCl at 100°C for 4 h. Basic fraction of the hydrolysate was subjected to paper chromatography using n-butanol/pyridine/water (6:4:3). The spot is glucosaminitol used as a standard.

day 4 (Fig. 2 D). The elution position (Kav=0.68) of the included peak, which seemed to be homogeneous because of its relatively sharp profile, remained unchanged until chase-day 10 (Fig. 2 F). As it is known that hyaluronic acid cannot be internalized by this type of cultured cell, it appeared that high-molecular-weight hyaluronic acid was extracellularly depolymerized into several fragments of constant size.

The molecular weight of the depolymerized hyaluronic acid was estimated to be about 40,000 by high-performance gel-permeation chromatography (data not shown). The depolymerized [^{3}H]hyaluronic acid was applied to a Sephadex G-25 column after incubation with β-N-acetylglucosaminidase. As shown in Fig. 3 A, no release of N-acetyl[^{3}H]glucosamine was observed. On the other hand, N-acetyl[^{3}H]glucosamine was released from the non-reducing terminals of hyaluronic acid by incubation with β-glucuronidase followed by β-N-acetylglucos-

aminidase (Fig. 3 B), suggesting that the non-reducing terminal of the depolymerized hyaluronic acid is glucuronic acid. The reducing terminal sugar of the depolymerized hyaluronic acid was identified by paper chromatography after reduction of non-isotopic hyaluronic acid with $NaB[^3H]_4$. As shown in Fig. 4, the reducing terminal sugar of the depolymerized hyaluronic acid was identified as N-acetylglucosamine.

DISCUSSION

Hyaluronic acid is a major glycosaminoglycan which is synthesized by human skin fibroblasts (12). It has been established, however, that hyaluronidase is absent from cultured human skin fibroblasts (4, 13, 14), and that hyaluronic acid is not internalized by these cells (5). Therefore, it is still not clear whether human skin fibroblasts are able to degrade hyaluronic acid. To resolve this uncertainty with regard to the degradation of hyaluronic acid in these cells, enzyme activities related to this process were measured (Table 1). It was found that activity of hyaluronidase, which is considered to be involved in the initial step of degradation of hyaluronic acid, was undetectable, corresponding to previous reports of others (4, 13, 14). Moreover, no activity of N-acetylhyalobiuronic acid-degrading β-glucuronidase (10), which is considered to participate in the final step of hyaluronic acid degradation, was detected. In addition, prolonged incubation with [^{3}H]hyaluronic acid produced no measurable uptake by the cultured cells (data not shown), as reported by Truppe et al. (5). These results suggest that hyaluronic acid is not degraded completely in cultured human skin fibroblasts.

Changes in the chain length of hyaluronic acid during incubation were then investigated. A pulse-chase experiment showed that the chain length of hyaluronic acid in the matrix fraction was unchanged, being of high molecular weight at least under monolayer culture conditions, whereas hyaluronic acid shed into the medium was depolymerized extracellularly (Fig. 2). Furthermore, prolongation of the chase period resulted in no oligosaccharides as shown by examination of "hyaluronidase" digests, and the chain length of the depolymerized products remained constant (Fig. 2). The molecular weight was estimated

to be about 40,000 by high-performance gel-permeation chromatography. The reducing and non-reducing terminal sugars were identified as N-acetylglucosamine and glucuronic acid, respectively. If this depolymerization occurs enzymatically, it is not yet explainable how the enzyme recognizes a product with such a relatively long chain size. It is evident, however, that endoglycosidase plays an important role in the degradation of chondroitin sulfate- and heparan sulfate proteoglycans (15-19). Similarly, the results of this investigation suggest that endoglycosidase, which cleaves the internal bond of hyaluronic acid, may be present in cultured human skin fibroblasts.

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