Hyaluronic acid in rabbit pericardial fluid and its production by pericardium

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High-M_r ($>2\times10^6$) hyaluronic acid (about 82 μg/ml) was found for the first time in rabbit pericardial fluid. Biosynthetic experiments with minced pericardium from rabbit showed that the high-M_r hyaluronic acid in the pericardial fluid was actively synthesized by the pericardium from [^{3}H]glucosamine.

Hyaluronic acid *Pericardial fluid* *Pericardium* *(Rabbit)*

1. INTRODUCTION

The functional roles of the pericardium and its pericardial fluid were confined to lubricating the moving surfaces of the heart and contributing mechanical support for the contraction of the ventricle [1]. However, we and other investigators have revealed that the pericardium is an active producer of prostacyclin and prostaglandins and plays an important role as extravascular source of prostacyclin in the pericardiac cavity [2–4].

Little is known about which glycosaminoglycans exist in the pericardial fluid of the heart, although the chemical composition and the results of the analysis of the protein constituents and of electrolytes of the pericardial fluid have been reported [5,6].

We report here that glycosaminoglycans, especially high-M_r hyaluronic acid (HA), were found for the first time in rabbit pericardial fluid and that the source of HA in the pericardial fluid was mainly pericardium.

2. MATERIALS AND METHODS

2.1. *Materials*

The following commercial materials were used: MEM from Grand Island Biological, NY; FCS from Boehringer Mannheim, FRG; streptomycin sulfate from Meiji seika, Tokyo; penicillin from Banyu Pharmaceutical, Tokyo; D-[6-^{3}H]glucosamine hydrochloride (22.6 μCi/mmol) from the Radiochemical Center, Amersham; chondroitinase AC (EC 4.2.2.5) from *Arthrobacter aureus*, chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*, hyaluronidase (EC 4.2.2.1) from *Streptomyces hyalurolyticus*, heparitinase (EC 4.2.2.8) from *Flavobacterium heparinum*, and ΔDi-4S, ΔDi-6S, and ΔDi-0S from Seikagaku Kogyo, Tokyo; pronase E (10^7 PU/g; 1 PU = 1 mmol tyrosine/min) from Kaken Kagaku, Tokyo; Sepharose CL-6B and CL-2B from Pharmacia, Uppsala; Toyo filter paper (no.51A) from Toyo Roshi, Tokyo; Shodex standrd P-82 (M_r markers) from Showa Denko, Tokyo.

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Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; FCS, fetal calf serum; HS, heparan sulfate; MEM, Eagle's minimum essential medium; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose

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2.2. *Preparation of rabbit pericardial fluid, plasma and pericardium*

Japanese white rabbits (3.0–3.5 kg) were anesthetized with sodium pentobarbital (39 mg/kg i.v.) and the pericardial fluid (250–300 μl) obtained from the cardiac cavity with a plastic syringe. It was centrifuged at 500 × *g* for 5 min at 4°C to remove any contamination of blood. About 3 ml pericardial fluid was obtained from 9–12 rabbits.

Blood was obtained from the carotid of rabbit with a plastic tube. The blood was kept for 30 min at room temperature and then centrifuged at 1000 rpm for 10 min. Plasma was separated and used for subsequent experiments.

Pericardium (50–100 mg wet wt) was removed from the rabbit heart and placed in ice-cold sterilized saline and then minced with scissors. The minced pericardium was used for subsequent experiments.

2.3. *Incubation of pericardium*

The minced pericardium was washed twice with MEM and incubated in 2 ml MEM containing 50 μCi [6-^{3}H]glucosamine in a flask under 5% CO_2 and 95% O_2 at 37°C. After 4, 8 and 12 h incubation, the medium (medium fraction) was removed from the flask and the pericardium (tissue fraction) rinsed with cold saline.

2.4. *Preparation and enzymic digestion of glycosaminoglycans*

Glycosaminoglycans from the pericardium fluid and medium and pericardium tissue were prepared after pronase digestion and cetylpyridinium chloride (CPC) treatment as described in [7]. Digestion with chondroitinase AC and chondroitinase ABC [8], *Streptomyces* hyaluronidase [9], and heparitinase [10] was described in previous papers.

2.5. *Descending paper chromatography*

The unsaturated disaccharides obtained from the isolated glycosaminoglycans by digestion with chondroitinase AC and ABC were spotted on Toyo no.51A filter paper (20 × 40 cm). After desalting in butan-1-ol/ethanol/water (13:8:4, by vol.) for 48 h, descending paper chromatography was carried out in butan-1-ol/acetic acid/1 M ammonia (2:3:1, by vol.) for 24 h at room temperature [11]. The resulting paper was cut into 1 cm bands and the segments extracted with water.

2.6. *Gel chromatography*

Gel chromatography was carried out on a Sepharose CL-6B column (1.4 × 65 cm) or Sepharose CL-2B (1.15 × 66 cm). The column was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl at a flow rate of 15 ml/h at 4°C. Fractions of 1.25 and 0.98 ml were collected in the case of Sepharose CL-6B and Sepharose CL-2B, respectively, and assayed for radioactivity and uronic acid.

2.7. *Assay for radioactivity, uronic acid and protein*

Radioactivity was measured with a liquid scintillation counter (Aloka LSC-900), with 8 ml Scintisol EX-H (Wako, Tokyo) containing 0.5 ml sample. The glycosaminoglycan content was determined as uronic acid by the modified carbazole method of Bitter and Muir [12]. Protein content was determined by the method of Lowry et al. [13].

3. RESULTS

3.1. *Characterization and identification of glycosaminoglycans in rabbit pericardial fluid and plasma*

The glycosaminoglycan concentration in rabbit pericardial fluid and plasma was determined as content of uronic acid (μg/ml). The uronic acid content in the pericardial fluid (36.6 ± 4.8 μg/ml, mean ± SD for 3 experiments) was about 14-times higher than that in plasma (2.6 ± 0.2 μg/ml, mean ± SD for 3 experiments). However, the protein content in plasma (50.5 ± 2.5 mg/ml, mean ± SD for 3 experiments) was about 2-times higher than that in the pericardial fluid (22.1 ± 1.9 mg/ml, mean ± SD for 3 experiments).

To characterize the molecular size of glycosaminoglycans obtained from rabbit pericardial fluid and plasma, gel chromatography on Sepharose CL-6B column was first carried out (fig.1). The majority of glycosaminoglycans isolated from the pericardial fluid (about 90% of the uronic acid) was eluted in the void volume and most of the excluded peak was digested with *Streptomyces* hyaluronidase, suggesting that the major component of glycosaminoglycans in rabbit

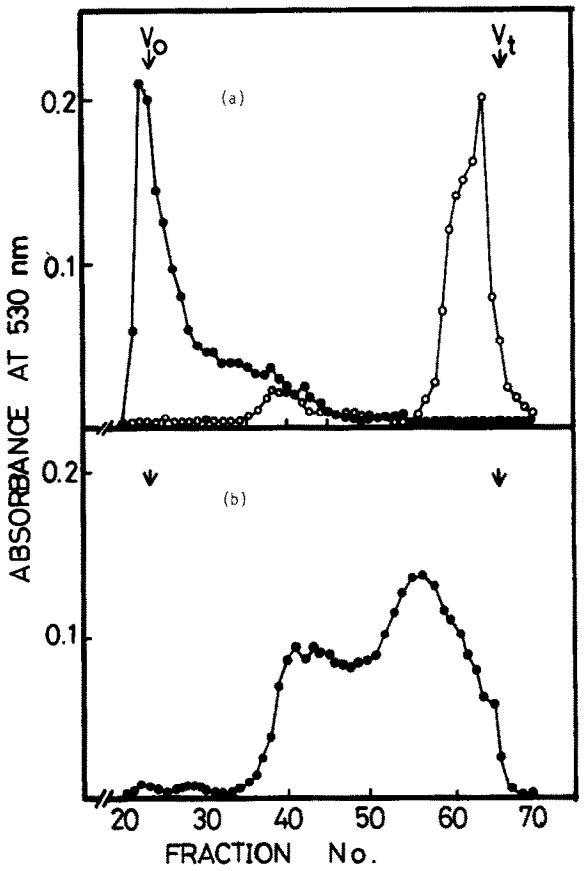


Fig.1. Elution profiles of glycosaminoglycans in rabbit pericardial fluid and plasma on Sepharose CL-6B. The glycosaminoglycans isolated from rabbit pericardial fluid and plasma were applied to a column (1.4 × 65 cm) of Sepharose CL-6B and eluted with 50 mM Tris-HCl buffer containing 0.15 M NaCl, pH 7.4. (a) Pericardial fluid (●—●), digested with *Streptomyces* hyaluronidase (○—○), (b) plasma (●—●). The arrows indicate (V_0) void volume and (V_t) total volume. The recovery of glycosaminoglycans after gel chromatography was more than 90%. For further details, see text.

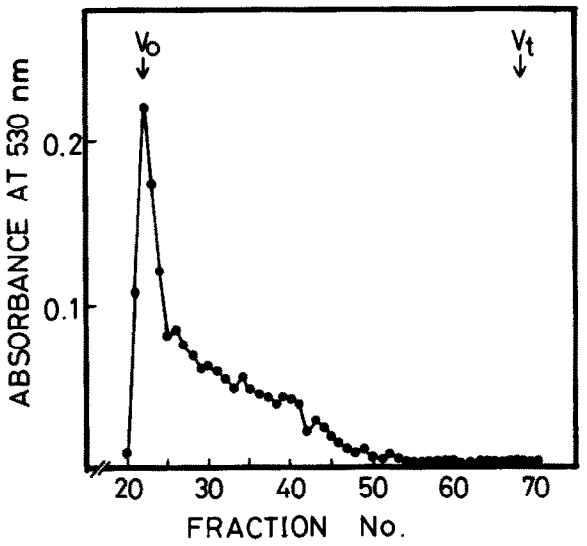


Fig.2. Elution profile of glycosaminoglycans in rabbit pericardial fluid on Sepharose CL-2B. The glycosaminoglycans isolated from rabbit pericardial fluid were applied to a column (1.15 × 66 cm) of Sepharose CL-2B and eluted with 50 mM Tris-HCl buffer containing 0.15 M NaCl, pH 7.4. The arrows indicate (V_0) void volume and (V_t) total volume. The recovery of glycosaminoglycans after gel chromatography was more than 90%. For further details, see text.

pericardial fluid was HA. The profile of glycosaminoglycans isolated from pericardial fluid on Sepharose CL-6B was quite different from that of glycosaminoglycans isolated from plasma. These results suggest no cross-contamination of plasma glycosaminoglycans in the pericardial fluid prepared. When the glycosaminoglycans from pericardial fluid were further subjected to a Sepharose CL-2B column, about 75% of the uronic acid was eluted in the void volume (fig.2). These results indicated that the major component of glycosaminoglycans in rabbit pericardial fluid was high-M_r HA ($M_r > 2 \times 10^6$).

The composition of glycosaminoglycans from the pericardial fluid and plasma was determined by descending paper chromatography of disaccharides obtained by the digestion of glycosaminoglycans with chondroitinase AC and ABC (table 1). More than 90% of glycosaminoglycans from the pericardial fluid was HA, while more than 85% of glycosaminoglycans from the plasma were CS and chondroitin low in sulfate. These results also indicated that the composition of glycosaminoglycans in rabbit pericardial fluid was clearly different from that of glycosaminoglycans in plasma.

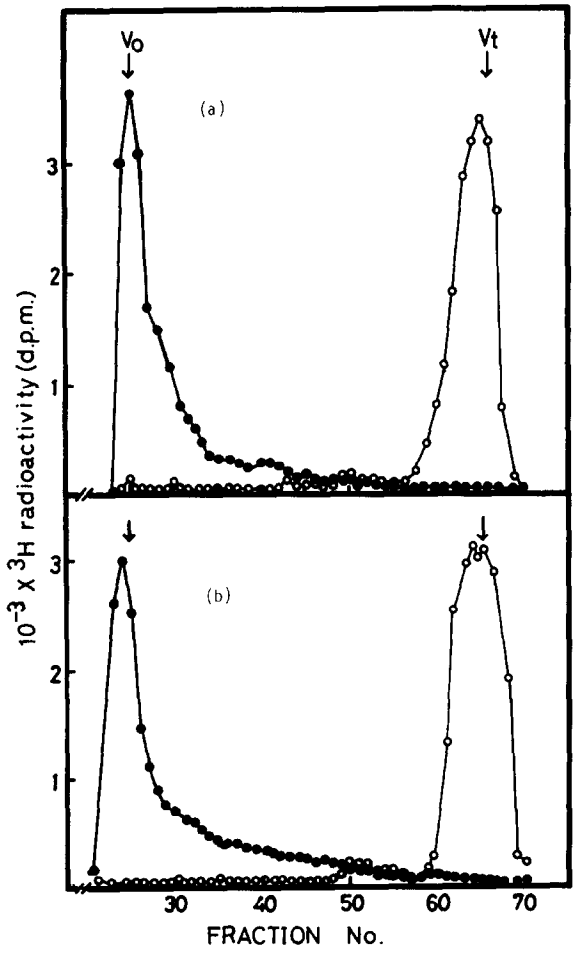
Thus, we can calculate the concentration of the high-M_r HA in the rabbit pericardial fluid as about 82 μg/ml (0.0082%, w/v), since the real concentration of HA is about 3-fold higher than the values of uronic acid (about 36.6 μg/ml) as the index of the glycosaminoglycan concentration and the per-

Table 1

Composition of glycoaminoglycans in rabbit pericardial fluid and plasma

	HA	HS	DS	CS		
				ΔDi-4S	ΔDi-6S	ΔDi-0S
Pericardial fluid	90.1	2.8	–	6.0	1.1	–
Plasma	14.9	–	–	34.2	5.9	45.0

The glycosaminoglycans isolated from rabbit pericardial fluid and plasma were digested with chondroitinase AC and ABC. The digested mixture was desalted and subjected to descending paper chromatography as described in the text. The composition was calculated from the absorbance at 232 nm of the extracted spots corresponding to the unsaturated disaccharide standards (ΔDi-4S, ΔDi-6S, and ΔDi-0S). All values given are the mean of 3 experiments and are shown as percentage of total glycosaminoglycans



cent uronic acid at the void volume of Sepharose CL-2B is about 75%.

3.2. *Characteristics and biosynthesis of glycosaminoglycans by pericardium*

To determine where HA in the pericardial fluid was synthesized, minced rabbit pericardium was incubated in MEM containing [^{3}H]glucosamine. The pericardium biosynthesized ^{3}H-labeled glycosaminoglycans during incubation times up to 12 h. When ^{3}H-labeled glycosaminoglycans synthesized by the pericardium were subjected to a Sepharose CL-2B column, they were eluted in the

Fig.3. Elution profiles of ^{3}H-labeled glycosaminoglycans biosynthesized in rabbit pericardium on Sepharose CL-2B. The ^{3}H-labeled glycosaminoglycans isolated from rabbit pericardium tissue and medium were applied to a column (1.15 × 66 cm) of Sepharose CL-2B and eluted with 50 mM Tris-HCl buffer containing 0.15 M NaCl, pH 7.4. (a) Pericardium tissue (●—●), digested with *Streptomyces* hyaluronidase (○—○), (b) medium (●—●), digested with *Streptomyces* hyaluronidase (○—○). The arrows indicate (V_0) void volume and (V_t) total volume. The recovery of glycosaminoglycans after gel chromatography was more than 90%. The elution profile shown is from a sample after 4 h incubation. The elution profiles at different incubation periods (8 and 12 h) were similar to this elution profile. For further details, see text.

Table 2

Composition of ^{3}H-labeled glycosaminoglycans biosynthesized in rabbit pericardium

	HA	HS	DS	CS		
				ΔDi-4S	ΔDi-6S	ΔDi-0S
Pericardium	92.7	1.6	–	2.7	1.8	1.2
Medium	96.0	1.0	–	1.0	0.9	1.1

The ^{3}H-labeled glycosaminoglycans isolated in pericardium tissue (about 80% of the total ^{3}H radioactivity) and its medium (about 20% of the total ^{3}H radioactivity) were digested with chondroitinase AC and ABC. The digestion mixture was applied to descending paper chromatography as described in the text. The composition of glycosaminoglycans was calculated from the ^{3}H radioactivity of the extracted spots corresponding to the unsaturated disaccharide standards (ΔDi-4S, ΔDi-6S, and ΔDi-0S). All values given are the mean of 3 experiments and are shown as percentage of ^{3}H total radioactivity

void volume (fig.3). Most of the excluded peak was digested with *Streptomyces* hyaluronidase. This suggests that most of the ^{3}H-labeled glycosaminoglycans biosynthesized by the pericardium was high-M_r HA.

The composition of ^{3}H labeled glycosaminoglycans biosynthesized was determined by descending paper chromatography of disaccharides obtained by chondroitinase AC and ABC digestion of ^{3}H-labeled glycosaminoglycans (table 2). More than 92% of the ^{3}H-labeled glycosaminoglycans was HA, the minor components being CS and HS. There was no significant difference in the components of radioactive glycosaminoglycans between the pericardium tissue and medium. In addition, supplementation of MEM with 10% FCS had no significant effect on the composition and M_r of ^{3}H-labeled glycosaminoglycans produced by the pericardium (not shown).

4. DISCUSSION

The possibility that the pericardium might secrete some substance which lubricates the moving surfaces of the heart has received little attention. This work is the first demonstration of the occurrence of high-M_r ($>2 \times 10^6$) HA (about 82 μg/ml) in the rabbit pericardial fluid (figs 1 and 2, table 1).

The biosynthesis experiments showed that the high-M_r HA was actively synthesized by the pericardium from [^{3}H]glucosamine (fig.3, table 2) and released into the pericardial fluid. When minced rabbit epicardium and myocardium were also incubated with [^{3}H]glucosamine in some paired incubations, the total incorporation of ^{3}H into glycosaminoglycans in the epicardium and myocardium was about 30 and about 12%, respectively, as compared with that (100%) in the pericardium (not shown). Therefore, the high-M_r HA can be considered to originate mainly in the pericardium, i.e. the serous parietal membranes which are lined with mesothelial cells [1], rather than an ultrafiltrate of blood serum.

It is probable that the high-M_r HA in the pericardial fluid could provide the heart tissue with resiliency and compliance when subjected to stretching or compressive forces.

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