Short Communications

Stimulation of Glycosaminoglycan Biosynthesis by Amyloid Fibrils

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Since amyloid-laden organs have an increased glycosaminoglycan content, the effect of amyloid fibrils on glycosaminoglycan metabolism by normal fibroblasts was examined. In comparison with controls, synthesis of glycosaminoglycans, primarily hyaluronic acid, was increased by an average of 48 and 93 % respectively when 0.1 and 1.0 mg of amyloid fibrils/ml was added to the cultures.

Amyloidosis is a disease of unknown aetiology characterized by the deposition in tissues of a unique fibrillar protein. Amyloid-laden tissues are marked, in addition, by an increase in glycosaminoglycan content (Brandt *et al.*, 1974). In amyloidotic livers (Linker *et al.*, 1958) and spleens (Bitter & Muir, 1966) the increase is attributable chiefly to heparan sulphate, although in other organs, e.g. heart, it may be attributable also to hyaluronic acid (Berenson *et al.*, 1969). Since the basis for the elevated glycosaminoglycan content of amyloid-laden tissues is unknown, the present study was designed to investigate the possibility that amyloid itself influences glycosaminoglycan formation. The results indicate that purified amyloid fibrils may stimulate synthesis of glycosaminoglycans by connective-tissue cells.

Human foetal skin fibroblasts (American Type Culture Collection, Rockville, Md., U.S.A.) were cultured in 90 % Dulbecco's Modified Eagle Medium (Grand Island Biological Co., Grand Island, N.Y., U.S.A.) containing 25 mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] buffer, pH 7.2, containing 10 % (v/v) of foetal calf serum (Grand Island Biological Co.), with penicillin (50 units/ml; Microbiological Associates, Bethesda, Md., U.S.A.) and streptomycin (50 µg/ml; Microbiological Associates) added. The cells were grown in CO₂+air (5:95), with the medium replaced every 48 h until they were confluent, when 4.5 ml of fresh medium containing 10 µCi of D-[6-³H]glucosamine/ml plus amyloid fibrils was added. The preparation of purified amyloid (a gift from Dr. Martha Skinner) was obtained from the liver of a patient with primary amyloidosis by a standard extraction and isolation procedure (Pras *et al.*, 1968). After 48 h the medium was decanted, and the cells were detached by treatment with trypsin and suspended in 0.1 M-sodium phosphate–0.15 M-NaCl, pH 7.4. Samples were re-

moved for cell counts and determination of cell viability (>95 % by Eosin Y exclusion). The remainder was manually homogenized and the glycosaminoglycan content of the cell homogenates was then determined as described below.

Glycosaminoglycans in media and in cell homogenates were precipitated with 4 vol. of ethanol, washed sequentially with 80 % (v/v) ethanol, ethanol and acetone, and dried *in vacuo*. Samples were then dissolved in 0.1 M-sodium acetate–0.15 M-NaCl, pH 5.0, and portions were digested with testicular hyaluronidase (300 EC units/ml; Miles Laboratories, Elkhart, Ind., U.S.A.) for 24 h at 37°C. Carrier hyaluronic acid (500 µg/ml) was added to digested and undigested samples, after which the glycosaminoglycans were precipitated with ethanol, washed and dried as described above. The dried precipitates were dissolved in the phosphate-buffered NaCl and added to a solution of toluene–Triton X-100 (2:1, v/v) containing 4.3 % (v/v) of Liquifluor (New England Nuclear Corp., Boston, Mass., U.S.A.). Radioactivities were counted in a Packard Tri-Carb liquid-scintillation spectrometer, with the results adjusted for variations in cell counts.

In every experiment, approx. 90 % of the ³H label was found in the medium with only about 10 % in the cell homogenate. In all cases a decrement of about 80 % was observed in the ³H c.p.m. radioactivity precipitable by ethanol after hyaluronidase digestion of the medium, indicating that most of the label had been incorporated into glycosaminoglycans. With increasing amounts of amyloid fibrils in the culture system increasing amounts of glycosaminoglycans were found in the media. Thus, on the basis of ³H loss after hyaluronidase digestion, incorporation of label into glycosaminoglycans, in comparison with controls, was increased by an average of 48 % (S.E.M. = ±7 %, *n* = 4) when amyloid was introduced

Table 1. Fractionation of glycosaminoglycans on cetylpyridinium-cellulose columns

After digestion of culture media with papain, glycosaminoglycans were isolated by precipitation with ethanol and dissolved in 5mM-Na₂SO₄. Samples were sequentially eluted from the column with 1% cetylpyridinium chloride, 0.3M-NaCl in 0.05% cetylpyridinium chloride and 6M-HCl (see the text). Duplicates of each sample were fractionated with virtually identical results; the data shown represent a single fractionation experiment. The percentage of the total glycosaminoglycans present in the medium was estimated from the sum of ³H d.p.m. radioactivities in the 0.3M-NaCl and 6M-HCl fractions. Abbreviation: CPC, cetylpyridinium chloride.

Sample	CPC fraction	Percentage of total ³ H d.p.m. radioactivity	Percentage of total glycosaminoglycans	Percentage of total hexosamine of fraction	
				Glucosamine	Galactosamine
Control	1% CPC	26.6	0	—	—
	0.3M-NaCl	65.5	89.3	92.2	7.8
	6M-HCl	7.9	10.7	23.2	76.8
Amyloid fibrils (0.1 mg/ml)	1% CPC	15.9	0	—	—
	0.3M-NaCl	79.2	94.2	100	0
	6M-HCl	4.9	5.8	31.3	69.7
Amyloid fibrils (1.0mg/ml)	1% CPC	19.3	0	—	—
	0.3M-NaCl	78.1	96.9	100	0
	6M-HCl	2.6	3.1	34.5	65.5

into the cultures at a concentration of 0.1mg/ml. When amyloid was introduced into the cultures at a concentration of 1.0mg/ml, incorporation of ³H into glycosaminoglycans was increased over controls by an average of 93% (S.E.M. = ±12%, *n* = 6).

In pulse-chase experiments, confluent cells were incubated in 4ml of medium containing 8μCi of D-[6-³H]glucosamine/ml with or without amyloid fibrils. After 24h the medium was decanted, the cells were washed and medium and wash were combined. Then 4ml of fresh medium was added and the incubation was continued for an additional 4, 12, 24 or 48h, after which the medium was decanted, the cells were rinsed and medium and wash were combined. The glycosaminoglycan content, adjusted for cell count, was derived from the decrement in ³H c.p.m. radioactivity precipitable by ethanol after hyaluronidase digestion. Results indicated that rates of degradation of glycosaminoglycans in the amyloid cultures and in controls were comparable, suggesting that the greater glycosaminoglycan content of fibroblast cultures exposed to amyloid fibrils was due to increased glycosaminoglycan synthesis.

To characterize the glycosaminoglycans, media were made 0.2M with respect to sodium acetate, pH5.2, and digested with crystallized (2×) papain (Kimmel & Smith, 1954). The glycosaminoglycans were then precipitated with ethanol, washed and dried as described above, and dissolved in 5mM-Na₂SO₄. Duplicate samples were then placed on a cellulose column (0.8cm×20cm) equilibrated with 1% (w/v) cetylpyridinium chloride and eluted sequentially with (a) 1% cetylpyridinium chloride containing 5mM-Na₂SO₄, (b) 0.3M-NaCl in 0.05% cetylpyridinium chloride and (c) 6M-HCl. The ³H

c.p.m. radioactivity of each fraction was determined as described above and, to account for unequal quenching in the various eluates, converted into d.p.m. radioactivity by internal standardization.

The 0.3M-NaCl and 6M-HCl fractions were made 8M with respect to HCl and hydrolysed under N₂ for 3h at 95°C, after which the acid was rapidly removed by boiling under N₂. Glucosamine and galactosamine in the hydrolysates were fractionated with a split-stream technique on a JEOL-5AH automated amino acid analyser and the proportions of the hexosamines were determined from the distribution of radioactivity in the isolated fractions.

Some 16–27% of the ³H eluted from the cetylpyridinium chloride column was present in the 1% cetylpyridinium chloride fraction (Table 1), in which proteins, peptides and keratan sulphate are eluted (Antonopoulos *et al.*, 1964), although the last-named is not produced by normal fibroblasts (Matalon & Dorfman, 1969). The ³H-labelled material not eluted by 1% cetylpyridinium chloride represents glycosaminoglycan (Antonopoulos *et al.*, 1964). Some 89–97% of the glycosaminoglycan was eluted by 0.3M-NaCl, which elutes hyaluronic acid from the cetylpyridinium chloride columns. Glucosamine represented 92–100% of the total hexosamine in the 0.3M-NaCl fraction, confirming that it contained predominantly or exclusively hyaluronic acid. Thus the increase in glycosaminoglycans that resulted from the introduction of amyloid into the cultures was essentially all due to increased hyaluronic acid synthesis.

The 6M-HCl fractions contained only about 3–11% of the ³H that had been incorporated into glycosaminoglycans (Table 1). In both control and amyloid-treated cultures galactosamine was the

predominant hexosamine in the 6M-HCl fractions, consistent with the presence of chondroitin sulphate or dermatan sulphate, whereas the small amount of glucosamine presumably represented heparan sulphate.

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