Altered proteoglycan synthesis by micromelial limbs induced by 6-aminonicotinamide

Appearance of abnormal forms of cartilage-characteristic proteoglycan (PG-H)

Atsushi HONDA,*† Satoshi KAZUNO,* Yo MORI,* Koji KIMATA† and Sakaru SUZUKI†

*Department of Biochemistry, Tokyo College of Pharmacy, Hachioji-shi, Tokyo 192-03, and †Department of Chemistry, Faculty of Science, Nagoya University, Nagoya 464, Japan

Since administration of 6-aminonicotinamide (10 μ g) to day-4 chick embryos *in ovo* was shown to induce micromelial limbs, biosynthesis of cartilage-characteristic proteoglycan-H (PG-H) as an important index of limb chondrogenesis was examined in day-7 normal and micromelial hind limbs by biochemical and immunological methods. (1) Metabolic labelling of the micromelial limbs with [6-³H]glucosamine and either [³⁵S]sulphate or [³⁵S]methionine, followed by analyses of labelled PG-H by glycerol density-gradient centrifugation under dissociative conditions, showed a marked reduction in the PG-H synthesis. (2) PG-H synthesized by the micromelial limbs was much lower than that synthesized by the normal limbs in the biosynthetic ratio of chondroitin sulphate to keratan sulphate and glycoprotein-type oligosaccharide, although no significant difference was observed in the immunological properties of these proteoglycans. (3) The degree of sulphation of chondroitin sulphates of PG-H was lowered in the micromelial limbs as judged by the increase of unsulphated disaccharide (Δ Di-OS) released by chondroitinase ABC digestion, although there were no significant differences between the normal and the micromelial limbs in the average molecular size ($M_r = 38000$) of labelled chondroitin sulphates of PG-H. (4) Addition of β -D-xyloside, an artificial initiator for chondroitin sulphate synthesis, to the micromelial limbs in culture recovered the incorporation of labelled glucosamine into chondroitin sulphate to that comparable with the normal control with β -D-xyloside, although the incorporation of [³⁵S]sulphate was lower in the micromelia than in the control with β -D-xyloside. These results suggest that the reduction in the biosynthesis of the PG-H as well as the production of altered forms of PG-H induced by 6-aminonicotinamide during a critical period of limb morphogenesis may be an important factor for the micromelia.

INTRODUCTION

6-Aminonicotinamide, a nicotinamide analogue, has been reported to be an experimental teratogen in mammals [1–4] and chickens [5]. When given in a dose of 10 μ g to chick embryos on day 4 of incubation, it causes micromelia as diagnosed by the disproportionate shortening of limbs [5]. Therefore, the developing limb is thought to be a primary target of 6-aminonicotinamide as a teratogen. Several workers have taken various histological and biochemical approaches for elucidation of the mechanism of micromelia caused by 6-aminonicotinamide [6–15]. Honda *et al.* [13] previously reported that it induced sequential molecular defects and concomitant abnormalities in glycosaminoglycan and proteoglycan biosynthesis in the limbs during the critical period of limb morphogenesis.

Recent studies by Suzuki and co-workers [16–20] have shown that chick-embryo epiphyseal cartilage synthesizes at least three proteoglycan species {termed PG-H [16, 17], PG-Lb [18] and PG-Lt [19] (also known as type IX collagen [19a])} which are analogous in having glycosaminoglycan side chains of the chondroitin (dermatan) sulphate type but are different from one another in regard to the structure of the core protein.

Using antibodies specific for these substances in indirect immunofluorescence, Shinomura *et al.* [20] demonstrated that PG-H and PG-Lb appear in a well-defined temporal and spatial pattern during early cartilage formation in the normal chick limbs. We therefore carried out comparative studies of the cartilage-characteristic proteoglycan, PG-H, produced by the normal and the micromelial hind limbs, using an antiserum prepared against PG-H.

We report here the abnormalities of PG-H biosynthesis in the micromelial limbs induced by 6-aminonicotinamide during limb formation of chick embryos.

MATERIALS AND METHODS

Materials

Freshly laid fertilized eggs of White Leghorn chickens were obtained from Saitama Prefectural Poultry Experiment Station, and were incubated at 38.5 °C in an electric incubator [13]. At day 4 (96 h incubation, i.e. Hamburger–Hamilton stage 24 [21]) 10 μ g of 6-aminonicotinamide in 50 μ l of sterilized distilled water was injected into the extra-embryonic coelom between the inner shell membrane and the vascular splanchnopleure.

Abbreviations used: PG-H, proteoglycan H; Δ Di-4S, 2-acetamido-2-deoxy-3-O-(β -D-glucosyl-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose; Δ Di-6S, 2-acetamido-2-deoxy-3-O-(β -D-glucosyl-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; Δ Di-OS, 2-acetamido-2-deoxy-3-O-(β -D-glucosyl-4-enepyranosyluronic acid)-D-galactose; e.l.i.s.a., enzyme-linked immunosorbent assay.

† To whom correspondence and reprint requests should be addressed.

Normal control eggs were given 50 μ l of sterile distilled water only. The eggs were incubated till day 7, then the hind limbs of day-7 chick embryos (corresponding to the Hamburger–Hamilton [21] stage 30–31) were excised from the root of the hind limbs of the 6-aminonicotinamide-treated or normal embryos, and were used for subsequent experiments.

The following commercial materials were used: D-[6- 3 H]glucosamine hydrochloride (40 Ci/mmol), D-[2- 3 H]mannose (272 mCi/mmol), L-[35 S]methionine (303 mCi/mmol), ACS II aqueous counting scintillant and NCS tissue solubilizer from Amersham International; [35 S]sulphuric acid (46 mCi/ml 0.05 M-HCl solution, carrier-free) from Japan Atomic Energy Institute, Tokyo, Japan; medium 199 and Eagle's minimum essential medium (Eagle's MEM, 'Nissui 5' without L-methionine) from Nissui Seiyaku Co. Ltd., Tokyo, Japan; streptomycin sulphate from Meiji Seika, Tokyo, Japan; penicillin from Banyu Pharmaceutical Co., Tokyo, Japan; 6-aminonicotinamide from Sigma Chemical Co.; guanidine hydrochloride from Nakarai Chemicals Ltd., Kyoto, Japan; pepstatin A from Peptide Institute Inc., Osaka, Japan; 4-methylumbelliferyl β -D-xylopyranoside from P-L Biochemicals, Inc., Milwaukee, WI, U.S.A.; sodium deoxycholate from Difco Laboratories, Detroit, MI, U.S.A.; bovine albumin, crystallized (purity 100%), from ICN Nutrition Biochemicals, Cleveland, OH, U.S.A.; pronase E from Kaken Chemicals Co., Tokyo, Japan; chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*, keratanase (EC 3.2.1.103) from *Pseudomonas* sp., endo- β -galactosidase (EC 3.2.1.103) from *Escheria freundii*, heparitinase (EC 4.2.2.8) from *Flavobacterium heparinum*, standard unsaturated disaccharide kit (Δ Di-4S, Δ Di-6S, and Δ Di-OS), and glycosaminoglycan preparations (human umbilical cord hyaluronic acid, whale cartilage chondroitin 4-sulphate, shark cartilage chondroitin 6-sulphate, pig skin dermatan sulphate, and bovine cornea keratan sulphate) used as reference standards from Seikagaku Kogyo Co., Tokyo, Japan; Sepharose CL-6B and Sephadex G-50 (fine) from Pharmacia; Toyo filter paper (no. 51A) from Toyo Roshi, Tokyo, Japan. All other reagents were of the highest grade commercially available.

Incubation experiments

The dissected hind limbs (normal limbs, about 300 mg wet wt./30 limbs; micromelial limbs, about 200 mg wet wt./30 limbs) from day-7 chick embryos were pre-incubated for 20 min in a 10 ml conical flask at 37 °C in an O₂/CO₂ (19:1) atmosphere with 2 ml of Medium 199 (sulphate concentration, 812 μ M; pH 7.2) containing antibiotics (penicillin 100 units/ml and streptomycin, 100 μ g/ml) and then incubated for another 2 h in the presence of 75 μ Ci of H₂ 35 SO₄ and 75 μ Ci of D-[6- 3 H]glucosamine hydrochloride. After the end of incubation, the limbs were rinsed with three 5 ml changes of ice-cold Medium 199 and the resulting labelled limbs were used for subsequent experiments.

In cases of the incubation experiments using D-[2- 3 H]mannose as a precursor, the labelling conditions were the same as described above except for the addition of 100 μ Ci of D-[2- 3 H]mannose to the medium.

In cases of the incubation experiments using L-[35 S]methionine, the dissected normal or micromelial hind limbs (about 100 limbs) from day-7 chick embryos were pre-incubated for 20 min in a 30 ml conical flask at

37 °C in an O₂/CO₂ (19:1) atmosphere with 15 ml of Eagle's minimum essential medium (Eagle's MEM, Nissui 5) without methionine containing antibiotic (kanamycin, 60 μ g/ml), and then incubated for another 24 h in the presence of 225 μ Ci of L-[35 S]methionine. After the end of incubation, the limbs were rinsed three times with the ice-cold Eagle's MEM and the resulting labelled limbs were used for subsequent experiments.

Incorporation of 3 H or 35 S radioactivity into the limbs was linear for up to 24 h of incubation.

Extraction of radioactive proteoglycans

Labelled proteoglycans were extracted from the normal or the micromelial hind limbs by a modification of the method described previously [13,16]. The labelled tissues were transferred to 5 vol. (about 1 ml) of cold extracting solution consisting of 4 M-guanidine hydrochloride/0.05 M-Tris buffer (pH 8.0) containing protease inhibitors: 10 mM-N-ethylmaleimide, 10 mM-disodium EDTA, 1 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin (this solution will be referred to as 'solution A'). The tissue suspension was frozen, kept at -70 °C in solid CO₂/acetone, and then thawed to 2 °C. The freezing/thawing procedure was repeated twice to facilitate the penetration of the solvent and the final suspension was kept at 0 °C for 48 h with gentle agitation. The mixture was centrifuged at 12000 rev./min for 30 min at 4 °C and the precipitation (tissue residue) was re-extracted in the same way. The supernatants were combined, and used as crude proteoglycan preparations for further separation of the cartilage-characteristic PG-H by zonal sedimentation on a glycerol gradient in 4 M-guanidine hydrochloride. About 80% of the total tissue labelled proteoglycans in either the normal or the micromelial limbs was extracted by this procedure.

Density-gradient ultracentrifugation of proteoglycan

Zone sedimentation of proteoglycans was performed as described previously [13,22]. An 0.8 ml aliquot of the crude extracted proteoglycan preparations with solution A was layered on a 15 ml gradient of glycerol [10–35% in solution A, prepared by Hitachi density gradient fractionator (DGF-U)] in a centrifugal polyallomer tube, at the bottom of which 0.5 ml of 50% glycerol in solution A had been placed. The tube was centrifuged in an SRP-28SA1 rotor in a Hitachi 65 P ultracentrifuge at 22500 rev./min at 20 °C for 28 h. Fractions (0.5 ml) were collected by a Toyo fraction collector with a Toyo mini-pump (TMP-15E), and the fractions were used for the measurement of radioactivity. The recovery of the total labelled proteoglycans in either the normal or the micromelial sample after the density-gradient centrifugation was more than 90%.

Preparation of anti-proteoglycan H antibody

An anti-PG-H antibody was prepared in accordance with the previously described methods [20,23]. Briefly, a rabbit was immunized using purified PG-H as an immunogen. About 600 μ g of PG-H was suspended in 50 μ l of phosphate-buffered saline and mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). This mixture was injected into a foot pad region of one leg of each rabbit. At two subsequent 2 week intervals, 300 μ g of the immunogen in 50 μ l of phosphate-buffered saline and an equal volume of incomplete Freund's adjuvant (Difco

Laboratories) were injected into the enlarged foot pads. The antibody titres were tested weekly by enzyme-linked immunosorbent assay (e.l.i.s.a.) [24]. The serum was collected one week after the second booster. The antiserum thus obtained had no cross-reactivity with PG-Lb and PG-Lt when examined by e.l.i.s.a. and radioimmunoprecipitation assay. The reactivity of PG-H to this antiserum was not altered by treatment of the antigen with chondroitinase ABC, but was completely abolished by trypsin digestion or alkali treatment, indicating that antigenic determinants are contained in the core protein. The titre of e.l.i.s.a was more than 30000-fold.

Immunoprecipitation of cartilage-characteristic PG-H with anti-PG-H serum

Labelled PG-H from the glycerol-density in 4 M-guanidine hydrochloride was precipitated with 3 vol. of ice-cold 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate. The precipitate was dissolved in 1 ml of 50 mM-Tris/HCl buffer (pH 7.4) containing 0.15 M-NaCl, 0.25% Triton X-100, 0.15% bovine serum albumin and protease inhibitors (10 mM-N-ethylmaleimide, 10 mM-disodium EDTA, 1 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin) (solution D) with gentle shaking at 4 °C overnight. To this mixture, 25 µl of anti-PG-H serum as a first antibody was added, and the mixture was incubated for 30 min at room temperature and then kept at 4 °C for 24 h. To this reaction mixture, 50 µl of anti-rabbit IgG (17.5 mg antibody/ml, Miles Laboratories Inc., Naperville, IL, U.S.A.) as a second antibody was added, and the mixture was incubated for 30 min at room temperature and then kept at 4 °C for 24 h. The immunoprecipitate was collected by centrifugation at 12500 rev./min for 15 min at 4 °C. The precipitate was washed twice with solution D, then washed with 50 mM-Tris/HCl buffer (pH 7.4) containing 0.25% Triton X-100, 0.25% sodium deoxycholate and protease inhibitors (washing buffer 1), and then washed with 50 mM-Tris/HCl buffer (pH 7.4) containing protease inhibitors (washing buffer 2). The resulting washed immunoprecipitate was dissolved in 1 ml of solution A and re-centrifuged by glycerol density-gradient centrifugation as above.

By these immunoprecipitation methods, 81–88% of the total radioactivity of the PG-H fractions from either the normal or the micromelial limbs was immunoprecipitated.

Preparation of radioactive carbohydrate chains from PG-H

PG-H samples were precipitated from water with 3 vol. of ice-cold 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate, treated with 1 ml of 0.05 M-NaOH/0.1 M-NaBH₄ and allowed to stand at room temperature for 24 h. The mixture was then adjusted to pH 7.5 by adding HCl and 0.1 vol. of 0.5 M-Tris/HCl buffer containing 25 mM-CaCl₂, pH 7.5. Pronase E was added to the mixture in two doses at 24 h intervals (dry tissue:Pronase E = 25:3, w/w), and proteolysis was carried out for 48 h at 45 °C in the presence of a small amount of toluene. The digest was deproteinized by adding trichloroacetic acid to a final concentration of 10%. The solution was kept at 4 °C overnight and centrifuged. The supernatant was neutralized with

NaOH and then lyophilized. This sample was designated as 'carbohydrate chains' from PG-H.

Gel chromatography

Molecular size distributions of the radioactive carbohydrate chains from PG-H of the normal or the micromelial limbs were determined by gel chromatography on a column (1.8 cm × 65 cm) of Sepharose CL-6B. The column was equilibrated and eluted with 0.2 M-pyridine/acetic acid (pH 5.0) at a flow rate of 6 ml/h at 4 °C. Fractions (3 ml) were collected and assayed directly for radioactivity. Whale cartilage chondroitin 4-sulphate (*M_r* 38000), as reference, was passed through the column to determine the molecular sizes of chondroitin sulphates from the normal and the micromelial limbs.

The low-*M_r* fraction (fraction L) of the carbohydrate chains from PG-H was further subjected to gel chromatography on Sephadex G-50. The column (1.0 cm × 65 cm) was equilibrated and eluted with 0.2 M-pyridine/acetic acid (pH 5.0) at a flow rate of 10 ml/h at 4 °C. Fractions (1 ml) were collected and assayed directly for radioactivity.

Chondroitinase ABC digestion and paper chromatography

The radioactivity distribution in non-sulphated, 4-sulphated, and 6-sulphated disaccharide units in labelled glycosaminoglycan preparations isolated from the normal or the micromelial hind limbs was determined as described by Saito *et al.* [25].

Identification of keratan sulphate

The materials from the void volume of the Sephadex G-50 column of the normal or the micromelial sample were digested with keratanase in 50 mM-Tris/HCl buffer (pH 7.2) for 60 h at 37 °C as described by Nakazawa & Suzuki [26] or heparitinase [27]. The keratanase digests were subjected to descending paper chromatography or to Sephadex G-50 column chromatography as described above. The materials from the void volume of the Sephadex G-50 column were also analysed by electrophoresis.

The electrophoresis was carried out on strips of cellulose acetate (Separax; Joko Sangyo Co., Tokyo, Japan) in 0.1 M-pyridine/0.47 M-formic acid (pH 3.0) at a constant current of about 1 mA/cm for 40 min [28]. The cellulose acetate strips with reference standards of hyaluronic acid, chondroitin sulphates, and dermatan sulphate were stained with 0.5% Alcian Blue in 3% acetic acid for 10 min and washed with 0.1% acetic acid and water [13]. The strips with the labelled materials were cut into 5 mm segments. Each segment was extracted with a mixture (1 ml) of NCS tissue solubilizer (Amersham)/water (9:1, v/v) at 50 °C for 2 h, and the resulting solution was counted in a liquid-scintillation counter. To identify further the two components separated on cellulose acetate strips, an aliquot of each component was digested with endo-β-galactosidase in 0.2 M-sodium acetate buffer (pH 5.8) at 37 °C for 24 h as described by Fukuda & Matsumura [29] or with chondroitinase ABC as described above.

Effects of β-D-xyloside on chondroitin sulphate synthesis

The conditions for metabolic labelling of limbs were the same as above except for the addition of 0.5 mM-4-

methylumbelliferyl β -D-xylopyranoside to the medium. After incubation for 2 h, the normal or the micromelial limbs were extracted with solution A as above. The medium samples were dialysed and lyophilized. The samples were dissolved in 1 ml of solution A and were subjected to Sepharose CL-6B chromatography to characterize the molecular size of the β -D-xyloside-induced chondroitin sulphates from the normal and micromelial limbs. The column (1.8 cm $\times$ 65 cm) was equilibrated and eluted with solution A at a flow rate of 6 ml/h at 4 °C. Fractions (3 ml) were collected and assayed for radioactivity

Immunofluorescence study of limbs

The preparation and fixation of the normal and micromelial limbs were performed as previously described by Shinomura *et al.* [20]. Hind limbs at stages 30–31 (day-7 chick embryos) were removed, embedded in Tissue-Tek II (Miles Laboratories) and quick-frozen

Fig. 1. Zone sedimentation profiles of [3 H]glucosamine- and [35 S]-sulphate-labelled proteoglycans synthesized by normal (upper panel) and micromelial (middle panel) limbs

[3 H]Glucosamine- and [35 S]sulphate-labelled proteoglycans, obtained from six hind limbs of the day-7 normal (control) or from 20 hind limbs of the day-7 6-aminonicotinamide treated (6-AN) chick embryos, were layered on the 15 ml glycerol gradient (10–35 %) in solution A and centrifuged at 22 500 rev./min for 28 h at 20 °C. Fractions (0.5 ml) were collected and assayed for radioactivity. Authentic cartilage-characteristic PG-H (about 1 mg wet wt./gradient) from epiphyseal cartilage of 12-day chick embryos was centrifuged under the same conditions as a reference (—) and the fractions were assayed for uronic acid [30] (lower panel). For further experimental details, see the text.

Fig. 2. Zone sedimentation profiles of [3 H]glucosamine- and [35 S]-sulphate-labelled proteoglycan immunoprecipitated with anti-PG-H serum from extracts of the normal (upper panel) and the micromelial (middle panel) limbs

Experimental conditions for the glycerol density-gradient centrifugation and immunoprecipitation are described in the legend to Fig. 1 and in the text. The peak of authentic PG-H from 12-day chick-embryo epiphyseal cartilage is indicated as a reference (—) in the lower panel. Abbreviation: 6-AN, 6-aminonicotinamide.

at solid CO_2 temperature. Frozen sections, 6 μm thick, were cut with a Cryostat microtome (American Optical Corp., Buffalo, NY, U.S.A.). Frozen sections on a glass slide were washed once with phosphate-buffered saline at room temperature to remove Tissue-Tek II, after which they were treated for 30 min at room temperature with anti-PG-H antibody. After several rinses in phosphate-buffered saline, the sections were incubated with fluorescein isothiocyanate-conjugated goat antibody to rabbit immunoglobulins (Miles-Yeda, Rehovot, Israel). After five phosphate-buffered saline washes, the preparation was mounted under a coverslip in glycerol/phosphate-buffered saline (9:1, v/v) and viewed with an Olympus BH₂RFL epi-illuminated fluorescence microscope (Olympus Kogaku Co., Tokyo, Japan).

Other analytical methods

Uronic acid was determined by the carbazole method of Bitter & Muir [30] with glucuronolactone as a standard.

RESULTS

Comparison of [35 S]sulphate- and [3 H]glucosamine-labelled PG-H by density-gradient centrifugation

Glycerol density-gradient centrifugation of the hind limb extracts from the normal and the micromelial

Fig. 3. Sepharose CL-6B chromatography of [^3H]glucosamine- and [^{35}S]sulphate-labelled carbohydrate chains released from PG-H synthesized by the normal (upper panel) and micromelial (lower panel) limbs

The pooled PG-H materials (indicated by the bars above the curve in Fig. 2) were treated with 0.05 M-NaOH/0.1 M-NaBH₄ and then digested with Pronase E. The resulting radioactive carbohydrate chains were subjected to a column (1.8 cm $\times$ 65 cm) of Sepharose CL-6B and eluted with 0.2 M-pyridine/acetic acid (pH 5.0). Fractions (3 ml) were collected and assayed for radioactivity. Abbreviation: 6-AN, 6-aminonicotinamide.

embryos from day 7 gave the sedimentation profiles shown in Fig. 1. The faster sedimenting proteoglycan which peaked at fractions 13–14 is the major component of the extract of normal limbs (six limbs). This peak corresponds to PG-H previously described in epiphyseal cartilages of 12-day chick embryos [16]. In the extracts of micromelial limbs (20 limbs), however, the predominant component is a slower sedimenting population which peaks at fractions 17–18. Both the PG-H fractions (indicated by the bars above the curve in Fig. 1) from the

normal and the micromelial limbs could be immunoprecipitated by the antibody against PG-H, showing that the main antigenic determinants were still present in the core protein of the micromelial proteoglycan. The precipitates were further subjected to glycerol density-gradient centrifugation, as shown in Fig. 2. The peak from the micromelial immunoprecipitate was again shifted to a slower sedimenting velocity region, compared with that of the normal control. The difference in sedimentation velocity may be due to a decrease in the proportion of carbohydrate chains compared with core protein in the micromelial PG-H (see below). However, the difference in sedimentation velocity between the control PG-H (Figs. 1 and 2, lower panel) and the control immunoprecipitated PG (Fig. 2, upper panel) may depend on the amount of proteoglycan loaded onto the gradients, because the control immunoprecipitated PG contains much smaller amounts of PG-H compared with that of the control PG-H (about 1 mg wet wt.).

To compare the activity of PG-H synthesis in the normal and micromelial hind limbs from day 7, the limbs were metabolically labelled with [^3H]glucosamine and either [^{35}S]sulphate or [^{35}S]methionine. The incorporation of [^3H]glucosamine and [^{35}S]sulphate into the PG-H fraction (indicated by the bars above the curve in Fig. 1) of micromelial limbs were about 25% (2.96×10^5 d.p.m./2 h per 100 mg wet wt.) and 20% (7.48×10^4 d.p.m./2 h per 100 mg wet wt.), respectively, of the normal control. The incorporation of [^{35}S]methionine into the core protein of the PG-H fraction of micromelial limbs was about 23% (116 d.p.m./24 h per 100 mg wet wt.) of that for the normal limbs, whereas that of [^{35}S]methionine into the extracted materials with solution A from the micromelial limbs was about 59% (3.52×10^5 d.p.m./24 h per 100 mg wet wt.) of the normal limbs. These results suggest that the biosynthetic activity of PG-H as a whole is much lower in the micromelial limbs than in the normal, and the activity of sulphation is impaired.

Comparison of the carbohydrate chains isolated from PG-H and the micromelial proteoglycan reactive with anti-PG-H

[^3H]Glucosamine- and [^{35}S]sulphate-labelled sugar chains, which were prepared after NaBH₄/alkali treatment and Pronase digestion of the normal and

Table 1. Alterations in the composition of unsaturated disaccharides derived from [^3H]glucosamine-labelled chondroitin sulphates synthesized by the normal or the micromelial limbs in the absence or presence of 4-methylumbelliperyl- β -D-xyloside

The [^3H]glucosamine-labelled chondroitin sulphates of PG-H from the normal or the micromelial limbs in the absence or presence of β -D-xyloside (0.5 mM) were prepared as described in the text. Portions of the labelled samples were digested with chondroitinase ABC and the digestion mixture was subjected to descending paper chromatography as described in the text. For calculations of the radioactivity distribution in disaccharide fractions, the sum of the disaccharides (d.p.m./disaccharide fraction) in each sample was taken as 100%. The values represent the mean of two experiments.

Chondroitin sulphates	Composition (d.p.m./disaccharide fraction) of:					
	$\Delta\text{Di-6S}$		$\Delta\text{Di-4S}$		$\Delta\text{Di-OS}$	
Normal fraction H	15948	(52%)	10564	(34%)	5952	(14%)
6-Aminonicotinamide-treated fraction H	1584	(45%)	956	(28%)	954	(27%)
Normal + xyloside (0.5 mM)	5896	(67%)	1998	(23%)	866	(10%)
6-Aminonicotinamide-treated + xyloside (0.5 mM)	2792	(63%)	572	(13%)	1048	(24%)

Fig. 4. Sephadex G-50 chromatography of the [^3H]glucosamine- and [^{35}S]sulphate-labelled low- M_r materials (fraction L) obtained from the Sepharose CL-6B column in Fig. 3.

The low- M_r materials (fraction L) from the Sepharose CL-6B column in Fig. 3 were subjected to a column (1.0 cm $\times$ 65 cm) of Sephadex G-50 and eluted with 0.2 M-pyridine/acetic acid (pH 5.0). Fractions (1 ml) were collected and assayed for radioactivity. Abbreviation: 6-AN, 6-aminonicotinamide. For further experimental details, see the text.

micromelial proteoglycan samples, were subjected to gel chromatography on Sepharose CL-6B (Fig. 3). Both the normal and micromelial samples yielded two distinct fractions, the high- M_r fraction (fraction H, K_{av} 0.30) and the low- M_r fraction (fraction L, K_{av} 0.76). There is a marked difference between the normal and micromelial samples in the ratio of fraction H to fraction L; 0.63 (^3H) or 0.73 (^{35}S) for the micromelial sample, compared with the values of 1.54 (^3H) or 3.27 (^{35}S) for the normal sample. Both fractions H corresponded in elution position to chondroitin 4-sulphate from whale cartilage (M_r 38 000; K_{av} 0.30).

Both fractions H were digested with chondroitinase ABC and the resultant disaccharides were analysed (Table 1). Compared with the normal, the micromelial sample showed a significant increase in the percentage of radioactivity incorporated into $\Delta\text{Di-OS}$ with a decrease in the radioactivity percentage incorporated into $\Delta\text{Di-6S}$ and $\Delta\text{Di-4S}$, consistent with the view that the micromelial proteoglycan carries low-sulphated chondroitin sulphates with almost the same molecular size (M_r 38 000) as the chondroitin sulphates of PG-H.

Both fractions L were separated into three peaks (K_{av} 0.00, 0.40 and 0.72, respectively) by gel chromatography on Sephadex G-50 (Fig. 4). However, in contrast with fraction H, there was no significant difference between the normal and the micromelial fraction L samples in terms of the elution profiles on G-50, although the amount of radioactivity incorporated into three peaks of the micromelial sample decreased as compared with that of the normal sample. Both the first peaks (K_{av} 0.00) of the void volume contained significant amounts of ^{35}S , but were not digested with heparitinase (results not shown). They contained two components (E-I and E-II)

Fig. 5. Cellulose acetate electrophoresis of [^3H]glucosamine-labelled carbohydrates (E-I and E-II) obtained from the Sephadex G-50 column of 6-aminonicotinamide (6-AN) sample in Fig. 4.

Electrophoresis was carried out in 0.1 M-pyridine/0.47 M-formic acid (pH 3.0) at a constant current of about 1 mA/cm for 40 min. The [^3H]glucosamine-labelled carbohydrates obtained from the void volume fraction of Sephadex G-50 (Fig. 4) were subjected to cellulose acetate electrophoresis before or after digestion with endo- β -galactosidase or chondroitinase ABC. Hyaluronic acid (HA), dermatan sulphate (DS), and chondroitin sulphates (CS) were used as reference standards. For further experimental details see the text.

as assessed by analyses on cellulose acetate electrophoresis before or after enzymic digestion. As shown in Fig. 5, E-I and E-II were digested with endo- β -galactosidase and chondroitinase ABC, respectively. Furthermore, E-I of the micromelial sample was also sensitive to keratanase as judged by the elution profiles of Sephadex G-50 chromatography, but it was not completely digested with keratanase (results not shown). These results indicate that major components of E-I and E-II represent keratan sulphate and chondroitin sulphate (low- M_r), respectively. Since the second peak, the intermediate size component, differed from the other two in being metabolically labelled with [$2\text{-}^3\text{H}$]mannose (results not shown), it should represent N-linked oligo-

Table 2. Effect of 4-methylumbelliferyl β -D-xyloside on the total incorporation of [3 H]glucosamine and [35 S]sulphate into medium and tissue chondroitin sulphate synthesized by the normal and the micromelial limbs

The normal or micromelial limbs were preincubated with or without 4-methylumbelliferyl β -D-xyloside (0.5 mM) in medium 199 for 20 min at 37 °C and then incubated with [3 H]glucosamine (75 μ Ci) and [35 S]sulphate (75 μ Ci) in Medium 199 for another 2 h at 37 °C. The medium fractions were dialysed against water. The limbs were extracted with solution A. Chondroitin sulphates were prepared from the medium and extract as described in the text. The values represent the mean of duplicate incubation experiments.

Chondroitin sulphates	$10^{-4} \times$ Incorporation into chondroitin sulphate (d.p.m./2 h per 10 limbs) of:	
	[3 H]Glucosamine	[35 S]Sulphate
Normal + xyloside (0.5 mM)	29.2	45.2
Normal fraction H	14.0	4.0
6-Aminonicotinamide- treated + xyloside (0.5 mM)	22.8	19.7
6-Aminonicotinamide- treated fraction H	2.2	0.2

Fig. 6. Sepharose CL-6B chromatography of radioactive chondroitin sulphate synthesized by the normal or micromelial limbs in the presence of 4-methylumbelliferyl β -D-xyloside

The medium samples of β -D-xyloside (0.5 mM)-induced chondroitin sulphate were subjected to a column (1.8 cm $\times$ 65 cm) of Sepharose CL-6B and eluted with 4 M-guanidine hydrochloride/0.05 M-Tris buffer (pH 8.0) containing protease inhibitors (solution A). Abbreviation: 6-AN, 6-aminonicotinamide. Fractions (3 ml) were collected and assayed for radioactivity. For further experimental details see the text.

saccharides, the presence of which has been demonstrated in PG-H by De Luca *et al.* [31] and Takahashi *et al.* [32]. The third peak, the smallest size component, corresponds in size to O-linked oligosaccharides. However, it was not characterized further in this study.

Fig. 7. Indirect immunofluorescent staining ($\times 20$) of the normal (upper panel) and the micromelial (lower panel) limbs of day-7 chick embryos

The sections were stained with anti-cartilage characteristic PG-H antibody. For further experimental details see the text.

Comparison of β -D-xyloside effects

4-Methylumbelliferyl β -D-xylopyranoside, an artificial initiator of chondroitin sulphate synthesis, was used to measure the capability of the micromelial hind limbs to synthesize chondroitin sulphate chains. Table 2 shows that addition of 4-methylumbelliferyl β -D-xyloside (0.5 mM) to the incubation medium of the micromelial limbs stimulated the total incorporation of [3 H]glucosamine into the medium plus the tissue glycosaminoglycan fraction to about 10 times that of the micromelial sample in the absence of β -D-xyloside, whereas the incorporation of [35 S]sulphate was about 99 times that of the micromelial sample in the absence of β -D-xyloside. In contrast, the total incorporation of [3 H]glucosamine into the medium plus the tissue glycosaminoglycan fraction of the normal limbs in the presence of β -D-xyloside was about twice that of the normal sample in the absence of β -D-xyloside, whereas that of [35 S]sulphate was about 11 times that of the normal sample in the absence of β -D-xyloside. To characterize the chondroitin sulphates synthesized in the presence of 4-methylumbelliferyl β -D-xyloside, the 3 H-labelled chondroitin sulphates were sequentially subjected to Sepharose CL-6B column chromatography and chondroitinase ABC digestion.

There were no significant differences between the normal and the micromelial limbs in the molecular size (K_{av} , 0.46) of chondroitin sulphate synthesized on 4-methylumbelliferyl β -D-xyloside (Fig. 6), although, as expected, the size of chondroitin sulphate chains (K_{av} , 0.46, in Fig. 6) appearing in the culture medium in the presence of β -D-xyloside was smaller than that of chondroitin sulphates (fraction H) (K_{av} , 0.30, in Fig. 3) synthesized in the absence of β -D-xyloside. Furthermore, chondroitinase ABC digestion showed that the percentage of radioactivity incorporated into Δ Di-OS was also higher in the micromelial hind limbs than in the normal in the presence of β -D-xyloside (Table 1). These results suggest that the micromelial limbs (day 7) have the potential ability to synthesize chondroitin sulphate that is normal in chain length but is low in sulphation degree.

Immunofluorescence study

The biochemical experiments described above suggest the appearance of the abnormal form of PG-H in the micromelial limbs induced by 6-aminonicotinamide. We therefore examined whether the micromelial limbs reacted with the antibody directed against the core protein of PG-H (Fig. 7). Both limb cartilages of day-7 normal and 6-aminonicotinamide-treated limbs were stained specifically with the antibody by an indirect immunofluorescence technique. However, in the 6-aminonicotinamide-treated limbs, the fluorescence of PG-H showed uneven distribution and the abnormal shape of the cartilage in the 6-aminonicotinamide-treated limbs was observed.

DISCUSSION

The results presented in this paper indicate that the PG-H synthesis in the micromelial limbs (day 7) induced by 6-aminonicotinamide reduces markedly compared with that in the normal limbs (Figs. 1 and 2), and that the PG-H synthesized by the micromelial limbs differs from that synthesized by the normal limbs in being lower in sulphation degree (Table 1) and in possessing smaller amounts of chondroitin sulphates relative to keratan sulphates and oligosaccharides (Figs. 3, 4 and 5). To confirm the decrease of PG-H synthesis in the micromelial limbs, the content of PG-H core protein was estimated by e.l.s.a. [24]. The contents of PG-H core protein in the micromelial limbs (day 7) were about 24% ($122.3 \pm 79.5 \mu\text{g}/30$ limbs, mean $\pm$ s.d. for three experiments) and about 47% ($35.2 \pm 5.9 \mu\text{g}/100$ mg wet wt. mean $\pm$ s.d. for three experiments), respectively, of the normal limbs. Furthermore, the core protein accounted for a lower proportion of the total proteins ($31.8 \pm 5.1 \mu\text{g}/100$ mg of protein, mean $\pm$ s.d. for three experiments) (A. Honda *et al.*, unpublished work). Therefore, the inhibition of PG-H core-protein synthesis in the micromelial limbs may be a specific effect of 6-aminonicotinamide rather than a reflection of a general inhibition of protein synthesis.

The marked reduction in the ratio of synthetic activity of chondroitin sulphates to keratan sulphates plus glycoprotein-type oligosaccharides of PG-H in the micromelial limbs could be due to the fact that 6-aminonicotinamide forms 6-aminonicotinamide adenine dinucleotide (6-amino-NAD) which replaces the normal coenzyme, NAD [33,34], thereby interfering with the

oxidation of UDP-glucose to UDP-glucuronic acid (UDP-glucose dehydrogenase reaction) necessary for chondroitin sulphate synthesis. However, as shown in Fig. 6 and Table 2, addition of the β -D-xyloside to the day-7 micromelial limbs restored the chondroitin sulphate synthesis to about 80% of the normal control level of [^3H]glucosamine incorporated. Therefore, it is unlikely that the appearance of the unusual PG-H in the micromelial limbs results from interference with the UDP-glucose dehydrogenase reaction.

The chondroitin sulphates (fraction H) of the PG-H synthesized by day-7 micromelial limbs were low in sulphation degree (Table 1). The addition of β -D-xyloside (0.5 mM) to the culture medium also caused increased synthesis of chondroitin sulphates of lower sulphation degree (Table 1). Furthermore, the low- M_r chondroitin sulphate (E-II, Fig. 5) of the micromelial limbs may be lower in sulphation degree as judged by the ^{35}S : ^3H ratio (E-II = 0.19; normal fraction H = 0.29), although the electrophoresis method used could not discriminate the different degrees of sulphation. Keratan sulphate (E-I, Fig. 5) of the micromelial sample was digested with endo- β -galactosidase, but not completely digested with keratanase, suggesting that the keratan sulphate of PG-H in the micromelial limbs may be lower in sulphation degree [35]. These phenomena can be explained by our previous findings [13] that 6-aminonicotinamide causes a significant reduction in both oxygen consumption and ATP levels, thereby leading to a decrease in formation of adenosine 3'-phosphate 5'-phosphosulphate. Decreased synthesis of this compound has been proposed for the mechanism of recessive mutant, brachymorphic (*bm/bm*) mice [36–39], the symptoms of which are similar to the micromelia induced by 6-aminonicotinamide. However, a decrease in sulphation would not explain why less proteoglycan (core protein) was present in the micromelial limbs. Besides undersulphation, other effects of 6-aminonicotinamide such as inhibition of PG-H core-protein synthesis and intracellular translocation or secretion must also be present in the micromelial cartilage. Particularly the interference with PG-H core-protein expression and the production of this abnormal form of PG-H at a critical stage of limb morphogenesis must be important factors for the growth retardation or disorder of cartilage. At this stage, whether this unusual form of PG-H produced in the micromelial limbs represents an intermediate or an incompleting form is unknown.

Several disproportionate dwarfisms have been described in both mice and chickens: *can/can* (cartilage anomaly) mouse [40], *cmd/cmd* (cartilage matrix deficiency) mouse [41], *cho/cho* (chondrodysplasia) mouse [42], *bm/bm* (brachymorphic) mouse [43], *cn/cn* (achondroplasia) mouse [43] and *nm/nm* (nanomelic) chicken [44]. Of these, *cmd/cmd* [23] mouse and *nm/nm* chick [45,46] seem to be most similar to the 6-aminonicotinamide micromelia in being defective in the synthesis of PG-H.

This study was supported in part by Grant-in-Aid for Special Project Research (no. 61106001) of Intracellular Processing of Biomolecules for the Expression of their Biological Functions from the Ministry of Education, Science and Culture of Japan. We thank Dr. Masahiko Iwama of Tokyo University of Agriculture for his helpful suggestions and advice. We also thank Mr. Isami Tsuboi of the Tokyo College of Pharmacy for his technical assistance in part of this work.

REFERENCES

1. Murphy, M. L., Dagg, C. P. & Karnofsky, D. A. (1957) *Pediatrics* **19**, 701–714
2. Pinsky, L. & Fraser, F. C. (1959) *Biol. Neonat.* **1**, 106–112
3. Schardein, J. L., Woosley, E. T., Peltzer, M. A. & Kaump, D. H. (1967) *Exp. Mol. Pathol.* **6**, 335–346
4. Turbow, M. M., Clark, W. H. & Dipaolo, J. A. (1971) *Teratology* **4**, 427–432
5. Landauer, W. (1957) *J. Exp. Zool.* **136**, 509–530
6. Caplan, A. I. (1972) *Dev. Biol.* **28**, 71–83
7. Seegmiller, R. E., Overman, D. O. & Runner, M. N. (1972) *Dev. Biol.* **28**, 555–572
8. Overman, D. O., Seegmiller, R. E. & Runner, M. N. (1972) *Dev. Biol.* **28**, 573–582
9. Seegmiller, R. E. & Runner, M. N. (1974) *J. Embryol. Exp. Morphol.* **31**, 305–312
10. Ritter, E. J., Scott, W. J. & Wilson, J. G. (1975) *Teratology* **12**, 233–238
11. Seegmiller, R. E., Horwitz, A. L. & Dorfman, A. (1980) *J. Embryol. Exp. Morphol.* **59**, 207–216
12. Sheffield, V. C. & Seegmiller, R. E. (1980) *J. Embryol. Exp. Morphol.* **59**, 217–222
13. Honda, A., Iwama, M., Umeda, T. & Mori, Y. (1982) *J. Biochem. (Tokyo)* **91**, 1959–1970
14. Iwama, M., Honda, A. & Mori, Y. (1983) *J. Pharmacobiodyn.* **6**, 613–620
15. Hirabayashi, Y., Mori, Y., Honda, A. & Yamada, K. (1986) *Connect. Tissue* **18**, 19–26
16. Oike, Y., Kimata, K., Shinomura, T., Nakazawa, K. & Suzuki, S. (1980) *Biochem. J.* **191**, 193–207
17. Oike, Y., Kimata, K., Shinomura, T., Suzuki, S., Takahashi, N. & Tanabe, K. (1982) *J. Biol. Chem.* **257**, 9751–9758
18. Shinomura, T., Kimata, K., Oike, Y., Noro, A., Hirose, N., Tanabe, K. & Suzuki, S. (1983) *J. Biol. Chem.* **258**, 9314–9322
19. Noro, A., Kimata, K., Oike, Y., Shinomura, T., Maeda, N., Yano, S., Takahashi, N. & Suzuki, S. (1983) *J. Biol. Chem.* **258**, 9323–9331
- 19a. Vaughan, L., Winterhalter, K. H. & Bruckner, P. (1985) *J. Biol. Chem.* **260**, 4758–4763
20. Shinomura, T., Kimata, K., Oike, Y., Maeda, N., Yano, S. & Suzuki, S. (1984) *Dev. Biol.* **103**, 211–220
21. Hamburger, V. & Hamilton, H. (1951) *J. Morphol.* **88**, 49–92
22. Kimata, K., Oike, Y., Ito, K., Karasawa, K. & Suzuki, S. (1978) *Biochem. Biophys. Res. Commun.* **85**, 1431–1439
23. Kimata, K., Barrach, H.-J., Brown, K. S. & Pennypacker, J. P. (1981) *J. Biol. Chem.* **256**, 6961–6968
24. Rennard, S. I., Kimata, K., Dusemund, B., Barrach, H. J., Wilczek, J., Kimura, J. H. & Hascall, V. C. (1981) *Arch. Biochem. Biophys.* **207**, 399–406
25. Saito, H., Yamagata, T. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1536–1542
26. Nakazawa, K. & Suzuki, S. (1975) *J. Biol. Chem.* **250**, 912–917
27. Linker, A. & Hovingh, P. (1972) *Methods Enzymol.* **28**, 902–911
28. Hata, R. & Nagai, Y. (1972) *Anal. Biochem.* **45**, 462–468
29. Fukuda, M. N. & Matsumura, G. (1976) *J. Biol. Chem.* **251**, 6218–6225
30. Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
31. De Luca, S., Lohmander, S., Nilsson, B., Hascall, V. C. & Caplan, A. I. (1980) *J. Biol. Chem.* **255**, 6077–6083
32. Takahashi, N., Ishihara, H., Tejima, S., Oike, Y., Kimata, K., Shinomura, T. & Suzuki, S. (1985) *Biochem. J.* **229**, 561–571
33. Dietrich, L. S., Friedland, I. M. & Kaplan, L. A. (1958) *J. Biol. Chem.* **233**, 964–968
34. Coper, H. & Neubert, D. (1964) *Biochim. Biophys. Acta* **82**, 170–172
35. Ito, M., Hirabayashi, Y. & Yamagata, T. (1986) *J. Biochem. (Tokyo)* **100**, 773–780
36. Orkin, R. W., Pratt, R. M. & Martin, G. R. (1976) *Dev. Biol.* **50**, 82–94
37. Schwartz, N. B., Ostrowski, V., Brown, K. S. & Pratt, R. M. (1978) *Biochem. Biophys. Res. Commun.* **82**, 173–178
38. Sugahara, K. & Schwartz, N. B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6615–6618
39. Pennypacker, J. P., Kimata, K. & Brown, K. S. (1981) *Dev. Biol.* **81**, 280–287
40. Johnson, D. R. & Wise, J. M. (1971) *J. Embryol. Exp. Morphol.* **25**, 21–31
41. Rittenhouse, E., Dunn, L. C., Cookingham, J., Cala, C., Spiegelman, M., Dooher, G. B. & Bennett, D. (1978) *J. Embryol. Exp. Morphol.* **43**, 71–84
42. Seegmiller, R., Fraser, F. C. & Sheldon, H. (1971) *J. Cell Biol.* **48**, 580–593
43. Lane, P. W. & Dickie, M. M. (1968) *J. Hered.* **59**, 300–308
44. Landauer, W. (1965) *J. Hered.* **56**, 131–138
45. McKeown, P. J. & Goetinck, P. F. (1979) *Dev. Biol.* **71**, 203–215
46. McKeown-Longo, P. J. & Goetinck, P. F. (1982) *Biochem. J.* **201**, 387–394

Received 9 October 1986/19 March 1987; accepted 9 June 1987