Regionally disturbed production of cartilage proteoglycans in malformed fetuses from diabetic rats

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Summary. Fetuses from normal and manifestly diabetic rats were obtained on pregnancy day 20. The fetuses from the diabetic rats were of normal or malformed morphology. Three tissue groups were dissected free; costal cartilage, the hard tissue of the rear, and of the frontal portion of the mandible. These tissues were maintained in vitro for 24 h during which time they were labelled with [³⁵S]sulphate. After the culture period the tissues were extracted with guanidine HCl and the resulting residues were further extracted with alkali. The culture medium was saved and its macromolecular content was compared to that of the extracts. The proteoglycans recovered in all extracts eluted at two distinct positions after chromatography on a Sepharose CL-2B column (peak I: K_{av} ~0.4, and peak II: K_{av} ~0.8), but the elution patterns were markedly different in extracts from various tissues. Thus, in rib cartilage, the majority of the labelled proteoglycans were

located in peak I (~90%) with no difference between extracts of fetuses from normal and diabetic pregnancies. In extracts of mandibular cartilaginous tissue from normal rat offspring the peak I percentage (60–80%) was lower than in the rib cartilage extracts. In the extracts from the frontal portion of malformed mandibles of fetuses of diabetic rats, the peak I percentage ($35 \pm 21\%$) was the lowest of all recorded and the only one to significantly differ from the other percentages in its (the frontal mandible) group. The results show an association between a congenital malformation, micrognathia, and a disturbance in the production of chondroitin sulphate proteoglycans in the malformed region.

Key words: Rat, teratology, diabetic pregnancy, mandible, congenital malformation, proteoglycans, proteoglycan synthesis, chondroitin sulphate, costal cartilage.

Previous studies have demonstrated that diabetes affects the metabolism of several connective tissue components [1], including the heparan sulphate [2] and chondroitin sulphate [3] proteoglycans. The full functional implications of these biochemical changes are at present unclear, but heparan sulphate alterations may hamper normal kidney performance [4], and a disturbed production of chondroitin sulphate proteoglycans may alter chondrocyte function [5]. It is also conceivable that maternal diabetes may induce proteoglycan disturbances in the offspring and thereby affect the maturation of chondrocytes leading to alterations of the cartilage model of the future osseous skeleton [6–9].

The fetuses of the rats used in the present study (denoted U rats, [10–11]) display skeletal malformations when the rat mother is manifestly diabetic, and we have previously shown that diabetic adult rats of this strain have an altered composition of chondroitin sulphate proteoglycans in their rib cartilage [3]. In addition, we have demonstrated that chondrocytes from the mandibular arch region of embryos of diabetic U rats are very sensi-

tive to a diabetes-like environment in vitro, since their development into mature chondrocytes is hampered when they are exposed to elevated ambient concentrations of glucose and β -hydroxybutyrate [12]. This finding suggests a specific and diabetes-associated metabolic vulnerability, which parallels the teratological sensitivity of this particular embryonic cell population.

The aim of the present investigation was to characterize the production of proteoglycans in mandibles from fetuses from diabetic U rats with and without mandibular malformations. The elution profiles were compared with the production pattern in rib cartilage, a tissue that does not show congenital malformations in rat diabetic pregnancy.

Materials and methods

Streptozotocin was a gift from the Upjohn Co., (Kalamazoo, Mi., USA). N-ethylmaleimide, benzamidine, ϵ -amino-n-caproic acid, DNP-alanine and chondroitinase ABC (chondroitin sulphate lyase,

Fig. 1. Percentage of total [^{35} S]sulphate-labelled macromolecules found in the culture medium from organ cultures of rib and mandibular cartilage from day-20-fetuses of normal (N) and diabetic (D) rat mothers. The fetuses of D rats show normal morphology (n), or exhibit mandibular malformations (m). Single determinations

EC 4.2.2.4) were obtained from Sigma Chemical Co., (St. Louis, Mo., USA). Inorganic [^{35} S]sulphate (carrier-free) was from The Radiochemical Centre, (Amersham, Bucks, UK). Sepharose CL-2B, Sepharose CL-6B, Sephadex G-50 and Blue Dextran were purchased from Pharmacia Fine Chemicals, (Uppsala, Sweden).

Induction of diabetes and pregnancy

Virgin female Sprague-Dawley rats (about 12 weeks old) of the sub-strain U [10, 11] were used for the experiment. The rats were given a single injection of 40 mg/kg streptozotocin [13] into the tail vein 4–6 weeks prior to the experiment. The streptozotocin was dissolved in 0.01 mol/l citrate buffer, pH 4.5. Animals with a serum glucose concentration in excess of 20 mmol/l one week after the injection were considered to be diabetic, the control animals were not injected and had a serum glucose concentration below 6 mmol/l.

Both control and diabetic females were caged overnight with non-diabetic males, the mating commenced in the diabetic animals 7–10 days after the injection of streptozotocin. The day sperm were found in a morning vaginal smear was designated gestational day 0.

In vitro labelling of proteoglycans

Fetuses from control and diabetic rats were carefully dissected out on day 20 of pregnancy, the fetuses from the diabetic rats were inspected and divided into two groups, normal and malformed. The costal cartilage and the hard tissue of the rear and frontal portions of the mandible (lower jaw) were dissected out and weighed. The sulphate labelling was performed in petri dishes (1 mCi [^{35} S]sulphate/dish) containing 2 ml Ham's F10 growth medium with HEPES at 37°C swirling at 90 rev/min. After 24 h the medium was removed and the tissues were immersed in 1 ml of extraction buffer [14] (4 mol/l guanidine HCl in 0.05 mol/l acetate buffer pH 5.8 containing 0.2% Triton X-100, 1 mmol/l N-ethylmaleimide, 0.1 mol/l ϵ -amino-n-caproic acid, 5 mmol/l benzamidine HCl and 10 mmol/l EDTA). After a further 24 h the extracts were dialysed against the extraction buffer. The remainder of the tissues were incubated in 2 ml 0.5 mol/l NaOH at 4°C for 24 h followed by neutralization. Medium and alkali extracts were dialysed against a 50 mmol/l Tris buffer, pH 8.0, prior to the estimation of the amount of macromolecular label per mg of tissue.

Chondroitinase ABC digestion of samples and subsequent gel chromatography on Sephadex G-50, were performed as previously described [15] and revealed that >95% of the labelled material in the extracts and the culture media was digestible, indicating that the label was present in chondroitin sulphate. Radioactivity was determined in a Beckman LS 3800 scintillation counter (Beckman Instruments Inc., Fullerton, Calif., USA).

Analytical methods

Proteoglycan size: The guanidine extracts were chromatographed in extraction buffer on a Sepharose CL-2B column (88 × 0.5 cm). Blue Dextran and DNP-alanine were used to indicate the void volume (V_0) and the total volume (V_t) of the column.

Polysaccharide chain length estimation: Sample extracts were dialysed against 0.2 mol/l NaCl and then digested overnight with papain (5% in 1 mol/l NaCl, 50 mmol/l EDTA, 50 mmol/l cysteine HCl, 25 mmol/l acetate, pH 5.5 at 37°C). After enzyme digestion the samples were chromatographed on a Sepharose CL-6B column (1 × 80 cm), eluted with 0.01 mol/l Tris buffer, pH 8.0, containing 0.15 mol/l NaCl and 0.1% sodium dodecyl sulphate (SDS). Blue Dextran and [^{3} H]H₂O were used as markers for void and total volume, respectively.

Chondroitin sulphate proteoglycan aggregation with hyaluronan: 200 μ l of guanidine sample extract together with 200 μ l (100 μ g/ml) high molecular weight hyaluronan were mixed and dialysed against 0.5 mol/l acetate buffer, pH 5.8 with added protease inhibitors (2 mmol/l N-ethylmaleimide, 1 mmol/l phenylmethylsulphonyl-fluoride, 1 μ g/ml pepstatin and 2 mmol/l EDTA) for 24 h and subsequently chromatographed on a Sepharose CL-2B column in the same buffer.

Statistical analysis

Significant differences between and within groups were estimated by one way analysis of variance (ANOVA) and the applied test was Fischer PLSD [16], at the 95, 99, and 99.9% significance levels. The statistical analyses were made using the StatView for Macintosh program.

Results

Less than 15% of the total [^{35} S]sulphate-labelled macromolecular material was left to be extracted by alkali. The total incorporation of label estimated as radioactivity per mg wet weight was 3–4 times higher in rib than in mandibular tissue (data not shown). However, the incorporation of radioactivity per mg wet weight was similar in each group, regardless of source of the tissue (i.e. the uptake of label per weight was of the same magnitude in mandibular tissue from offspring of normal rats, as in tissue from non-malformed and malformed fetuses of diabetic rats). The release of proteoglycans into the medium was highest for mandibles from malformed fetuses, as estimated by the radioactivity found in the medium in percent of total macromolecular labelled material in extracts and medium (Fig. 1). The composition of chondroitin sulphate proteoglycans in cartilaginous tissue from different anatomical locations showed pronounced variations in fetuses with mandibular malformations. Thus, in cartilage extracts from fetal ribs the large (partly aggregating) species

Fig. 2. Cartilage extracts from a malformed fetus chromatographed on Sepharose CL-2B in 4 mol/l guanidine HCl, separation of proteoglycans into two categories, peak I (K_{av} ~0.4) and peak II (K_{av} ~0.8). **A** Rib cartilage extract. **B** Extract from back part of mandible. **C** Extract from front part of mandible

Fig. 3. Percentages of extracted proteoglycans from different cartilages eluting in peak I when chromatographed in extraction buffer on Sepharose CL-2B. Normal (N) and diabetic (D) rat mothers, fetuses with normal (n) and malformed (m) mandibular morphology. There is no significant difference at 95% in analysis of variance (Fischer PLSD) within the groups 'ribs' or 'back part of mandible' whereas the front part of malformed mandibles differs from the other members of its group at 99.9%

dominated (Fig. 2 A). In extracts from mandibular tissue there was a higher proportion of smaller, mainly non-aggregating, proteoglycans present (Fig. 2 B and 2 C). The relative proportion of large (peak I) proteoglycans was highest (~90%) in rib cartilage, slightly lower in the rear portion of the mandible, and further decreased in the ex-

tracts from frontal portions of malformed mandibles (Fig. 3). In tests of significance in the difference in relative proportion of peak I proteoglycans by analysis of variance no significant differences were found within the groups 'ribs' and 'back part of mandible' ($p > 0.05$, ANOVA) whereas in the 'front part of mandible' group the malformed mandibles were significantly different from non-malformed mandibles whether these were from fetuses of control or diabetic rats ($p < 0.001$, ANOVA, [Fig. 3]). (If the two lowest values in the malformed group were excluded the percentage mean remained significantly different from all other values in the group ($p < 0.05$, ANOVA)).

Proteoglycans recovered from the medium of mandibular organ cultures contained similar amounts of peak I material as the guanidine HCl extracts while the proportion aggregating proteoglycans were considerably lower (~10% of total vs ~30–40% in extracts). No differences between medium proteoglycans from frontal and rear portions of the mandibles or normal and malformed mandibles were noted (data not shown).

When guanidine HCl extracts from front parts of the mandibles were incubated with hyaluronan under associating conditions and subsequently chromatographed, a large proportion of the proteoglycans bound to hyaluronan and consequently eluted early (Fig. 4 A and B). The V_o peaks from this associative chromatography were collected and re-chromatographed under dissociative conditions. The elution profiles differed markedly; the aggregating proteoglycans from the non-malformed mandibles eluted in a single peak of large proteoglycans (Fig. 4 A), whereas the aggregating proteoglycans from the malformed mandibles eluted in two peaks as large and small proteoglycans (Fig. 4 B).

Fig. 4. Cartilage guanidine HCl extracts from the front portion of non-malformed **A** and malformed **B** mandible, both from 20-day-old fetuses from diabetic rat mothers. Chromatography on Sepharose CL-2B in associative buffer after incubation with hyaluronan (—). The material under the bars was pooled and re-chromatographed in dissociative extraction buffer (---)

The polysaccharide chain lengths of the chondroitin sulphate proteoglycans in the guanidine HCl extracts were estimated by chromatography on Sepharose CL-6B after papain digestion of the core proteins, the K_{av} values (range: 0.40–0.44) were found to be unaffected by anatomical region and maternal metabolic state (data not shown).

Discussion

In the rat model for diabetic pregnancy used in the present investigation, the teratological period for the skeleton has been shown to appear as early as during gestational days 6–10, corresponding to gestational weeks 2–4 in human pregnancy [17]. The most frequent skeletal malformation in the offspring of the diabetic rats is a grossly underdeveloped mandible, i.e. micrognathia [10]. It is of considerable interest, therefore, that the estimated teratological time period [18], gestational days 6–10, both precedes and includes the migration of neural crest cells to the mandibular arch region in the embryo. This migratory process provides the precursor cell population of the future cartilage of Meckel [19, 20] and, subsequently, the mandibular bone [21].

While the excised rib cartilage is undoubtedly cartilage, clear and uncalcified, the mandibular hard tissue studied is more ambiguous. This tissue is partly calcified and consists of regions of Meckel's cartilage, partly resorbed and partly calcified, there are tooth forming regions, and the membrane-bone regions which will also form secondary cartilages. Perhaps it should largely fall under the heading of condroid bone [22]. The proteoglycans extracted from this tissue are, however, mainly cartilage type proteoglycans [3], clearly distinct from tooth [23] or bone [24] proteoglycans.

The aim in the present study was to search for characteristic alterations in the metabolism of proteoglycans in fetal tissue from malformed regions. We have here demonstrated that malformed mandibles exhibit a disturbed metabolism of chondroitin sulphate proteoglycans. The alteration appears closely associated with the malformation since it is not present in rib cartilage or in non-malformed mandibles from fetuses of diabetic rats. The disturbance is in part similar to that previously described for cartilage in adult diabetic rats comprising a shift in the biosynthesis pattern from large to small matrix proteoglycans [3]. Also, there is a similarity to (pre)chondrocyte cultures [25], a higher percentage of proteoglycans being released to the medium from cultures from sensitive/malformed regions. A further indication of disturbed proteoglycan metabolism was the discovery of a class of small aggregating proteoglycans in the guanidine extract of malformed mandibles. This suggests an abnormal catabolism of the large cartilage-specific proteoglycan, aggrecan, in the malformed regions of the mandible and may be important in the pathogenesis of the developmental alterations of the tissue.

The fetal mandible is moulded on the cartilage of Meckel which in turn is formed by neural crest cells that invade this anatomical region during the fourth week of

embryogenesis in humans (corresponding approximately to gestational day 10 in rats). Cell migration is regulated by amounts and relative proportions of extracellular matrix proteins such as fibronectin and laminin [26], as well as proteoglycans [27]. While low concentrations of laminin have been demonstrated to be stimulatory for cell motility in vitro, the motility of neural crest cells was strongly reduced at higher concentrations [28]. Interestingly, increased concentrations of laminin have been demonstrated in embryos of diabetic rats [29].

It is premature to make extensive conclusions regarding causal relationships from the combined findings in this and previous studies of defective chondrogenesis in a diabetic environment, but if the migration of the initial neural crest cell population is inhibited, a less than sufficient number of chondrogenic cells may be present in the mandibular arch (presumably less dense the further the distance from their origin) to allow chondrogenesis to proceed normally. In addition, the role of altered epithelial-mesenchymal interaction, leading to changes in expression of critical genes and gene families, should be taken into account [30].

In conclusion, the results indicate an association between a cartilage-related congenital malformation, micrognathia, and a disturbance in the metabolism of chondroitin sulphate proteoglycan in the malformed anatomical region.

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