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Short Communication

Glycosaminoglycans in early chick embryo

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ABSTRACT The developmental profile of glycosaminoglycans (GAGs) were examined by cellulose acetate electrophoresis and high performance liquid chromatography in the early chick embryo from late blastula (stage XIII$^+$) to early somite developmental stages (stage HH7-9). Sulphated GAGs were present from the earliest stages. They were more abundant than the non-sulphated forms and showed stage-related changes. Chondroitin sulphate and especially dermatan sulphate appeared to be the predominant GAGs in embryos at stage XIII$^+$. Dermatan sulphate was about three times as abundant as chondroitin sulphate at stage XIII$^+$. In contrast, embryos at the definitive streak stage (stage HH4) produced about twice as much chondroitin sulphate as dermatan sulphate. At the head process stage (stage HH5), the level of chondroitin sulphate was reduced and its relative content in the embryo was about the same as dermatan sulphate. Levels of dermatan sulphate were more than five times those of heparan sulphate from stage XIII$^+$ through to stage HH5 and three times more at stage HH7-9. The 4- and 6- sulphation of chondroitin sulphate increased 14- and 10-fold respectively, from stage XIII$^+$ to stage HH 7-9. The sulphation pattern of chondroitin sulphate had a Δdi-4S:Δdi-6S molar ratio ranging from 4 to 8:1 and a Δdi-4S:Δdi-OS molar ratio ranging from 9 to 16:1 and was developmentally regulated. Thus, chondroitin sulphate in the early chick embryo was sulphated predominately in the 4-position in all stages studied. The presence of both 4- and 6-sulphated disaccharides in chondroitin sulphate indicated that both 4 and 6 sulfotransferases were active in the early embryo. Hyaluronate and sulphated GAG content increased markedly at gastrulation when the first major cellular migrations and tissue interactions begin.

KEY WORDS: *glycosaminoglycans, extracellular matrix, morphogenesis, chick embryo*

Glycosaminoglycans (GAGs) are a major class of components of embryonic extracellular matrix, occur as large unbranched polymers of repeating disaccharides and, with the possible exception of hyaluronate, are covalently linked to a core protein to form the proteoglycans. The structure of GAGs is variable due to various factors such as the degree of sulphation, the position of sulphates in repeating disaccharide units, the presence or absence of iduronic acid, the type of hexosamine present and the structure of the region that links GAGs to protein cores (Reviewed by Toole, 1991; Bandtlow and Zimmermann, 2000). In many systems, GAGs both in the interstitial spaces between cells and at the cell surface act key roles in regulating adhesion, proliferation, migration and morphogenesis particularly because of their polyanionic nature.

The importance of GAGs as compounds necessary for normal morphogenesis in early development was recognised in scores of work (Reviewed by Harrisson, 1989 and Zagris, 2000). In the chick embryo, injecting ^{35}S-sulphate in unincubated eggs and autoradiography after different incubation times showed that sulphated GAGs were present from the earliest stages and that regional differences of their distribution occurred from late gastrulation on (Johnston and Comar, 1957). Incorporation studies of ^{35}S-sulphate or ^{3}H-glucosamine and autoradiography during elongation of the primitive streak of chick embryo (stage HH3$^+$) showed uniform labelling in the forming germ layers and strong labelling of the basement membrane on the ventral side of the epiblast and in the primitive streak where the epiblast cells de-epithelialize in migrating cells (Vanroelen and Vakaet, 1981; Harrisson, 1989); most autoradiographic labelling was sensitive to degradation by GAG-degrading enzymes and by nitrous acid suggesting the presence of GAGs of which hyaluronate seemed

Abbreviations used in this paper: CS, chondroitin sulphate; DS, dermatan sulphate; Δdi-4S, 4-sulphated Δ-disaccharide; Δdi-6S, 6-sulphated Δ-disaccharide; Δdi-OS, non sulphated Δ-disaccharide; Δdi-OS_{CS}, non sulphated Δ-disaccharide of chondroitin sulphate; Δdi-OS_{HA}, Δ-disaccharide of hyaluronate; GAG, glycosaminoglycan; HPLC, high performance liquid chromatography; HA, hyaluronate.

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to be the predominant. Incorporation of ^{35}S-sulphate and autoradiography during shortening of the primitive streak at the head process stage (stage HH5), showed labelling of the three germ layers but an intense amount of label was detected at the level of the developing notochord; enzymatic characterization of the GAGs produced demonstrated that at least chondroitin sulphate and heparan sulphate were present in the labelled tissues (Vanroelen and Vakaet, 1981). When explanted trunk segments from 3-day old chick embryos were labelled with ^{35}S-sulphate, the neural tube produced chondroitin sulphate, heparan sulphate and hyaluronate while the notochord produced chondroitin sulphate and heparan sulphate but not hyaluronate (Hay and Meier, 1974). Ultrastructural localisation of GAGs by staining and by autoradiography revealed that different embryonic tissues make different relative amounts of sulphated GAGs and hyaluronate and these differences may reflect their morphogenetic significance during embryogenesis (Vanroelen and Vakaet, 1981; reviewed by Harrisson, 1989). Staining distribution was presumed to be a semi-quantitative reflection of biochemical distribution. The aim of the present study was to analyse the types and relative proportions of GAGs in chick embryo from blastula (stage XIII) to early somite development (HH7-9). The developmental profile and the expression patterns of GAGs were analysed by cellulose acetate electrophoresis and by high performance liquid chromatography (HPLC).

In our present work, a GAG fraction from chick embryos was obtained by removing lipids and proteins by the use of solvents and proteolytic enzymes, respectively. GAGs were subjected to zone electrophoresis before and after selective degradation by highly specific enzymes. Aliquots from the enriched GAG fraction from embryos at stages XIII$^+$ (late blastula, lane 1), HH3 (mid gastrula/intermediate streak, lane 2), HH4 (late gastrula/definitive primitive streak, lane 3), HH5 (early neurula / head process, lane 4) and HH7-9 (6 somites, lane 5) were analysed by cellulose acetate electrophoresis (Fig. 1A). For comparison a mixture of GAG reference standards were electrophoresed simultaneously (Fig. 1, lane 6). In ascending order of mobility based on their sulphated content per disaccharide ratio, the bands were identified as hyaluronate (HA), heparan sulphate (HS), dermatan sulphate (DS) and chondroitin sulphate (CS). Hyaluronate which is a non-sulphated GAG represented the slowest moving band while heparin has the highest degree of sulphation and migrated ahead of CS (Fig. 1). Chondroitin sulphate and especially DS appeared to be the predominant GAGs in embryos at stage XIII$^+$ (Fig. 1A, lane 1). During stage HH3, DS and CS were the predominant GAGs and HS and HA were also present in large amounts (Fig 1A, lane 2). Chondroitin sulphate, DS, HS and HA were prominent and heparin, which migrated faster than CS, was intense at stage HH4 (Fig. 1A, lane 3). At the head process stage, heparin appeared to be intense while CS, DS, HS and HA were present in large amounts (Fig. 1A, lane 4). At stage HH7-9 presence of CS, DS, HS and HA was marked (Fig. 1A, lane 5).

The GAG types were estimated by selective degradation with heparin lyases I/III which specifically degrade HS and heparin and with chondroitinase AC II which specifically degrades HA and CS (containing D-glucuronic acid units), but not DS (containing L-iduronic acid units) (Fig. 1B). In Fig. 1B, the residue resistant to chondroitinase AC II and to heparin lyases that was present was assumed to be DS and keratan sulphate. When in the enzyme cocktail described above chondroitinase AC II was replaced by chondroitinase ABC which degrades HA and CS and DS, DS was also degraded (Fig. 1C). The residue resistant to chondroitinase ABC and heparin lyases used to analyse the GAGs could be assumed to be keratan sulphate and / or an unidentified component(s) (Fig. 1C). This could point to possible structural changes in the GAG chains during embryonic development. In Fig. 1 B,C, lane numbers correspond to embryos at the stages as indicated in Fig. 1A.

The areas under the curves in the densitometric patterns for individual GAGs were obtained from the electrophoretogram in Fig. 1A and the amount of CS, DS and HS was calculated using the Scion Image PC (Table 1). There is an increase in the biosynthesis of GAGs during the course of development from blastula to the early somite stages in chick embryo. Dermatan sulphate was increased about 3 fold from stage XIII$^+$ to stage HH3, persisted at about the same level at stages HH4 and HH5 and then increased another 3 fold by stage HH7-9. Chondroitin sulphate was increased more than 5 fold by stage HH3 and 16-, 8- and 20-fold by stages HH4, HH5 and HH7-9, respectively, compared to the amount detected at stage XIII$^+$. Dermatan sulphate was more than 2 fold predominant to CS at stage XIII$^+$. Then, it was striking to note that embryos at the definitive streak

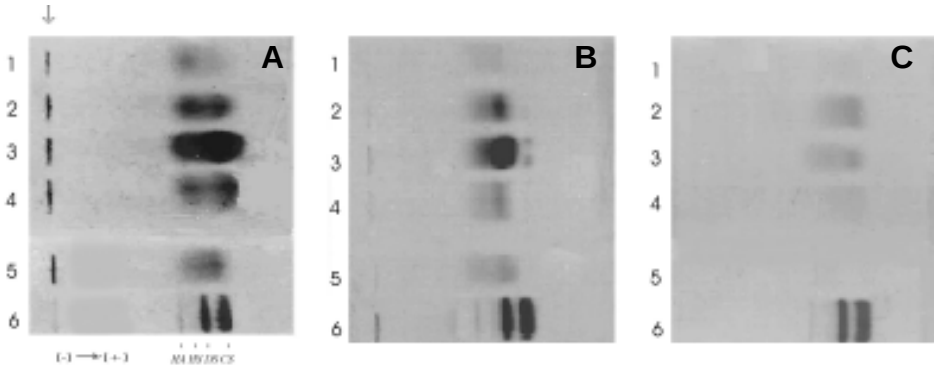

Fig. 1. Separation of glycosaminoglycans (GAGs) isolated from early chick embryos. *The GAG fraction from chick embryos at various stages was obtained by removing lipids and proteins. Glycosaminoglycans from embryos at stages XIII$^+$ (late blastula, lane 1), HH3 (mid gastrula/intermediate streak, lane 2), HH4 (late gastrula/definitive primitive streak, lane 3), HH5 (early neurula / head process, lane 4) and HH7-9 (6 somites, lane 5) were separated by cellulose acetate electrophoresis. Identification of GAGs was according to electrophoretic separation of commercial GAG standards (Sigma, Saint Louis, USA) (lane 6). Part of the GAG fraction from embryos at stages indicated in* **(A)** *was treated either with chondroitinase AC II* **(B)** *or with chondroitinase ABC* **(C)** *and heparin lyases I/III and the GAG types were estimated by cellulose acetate electrophoresis. Notation of lanes in (B) and (C) as in (A).Electrolyte: 0.1 M pyridine, 0.47 M formic acid (pH 3.1). Constant current: 2 mA/cm for 45 min. The horizontal arrow marks the direction of mobility; the vertical arrow marks the origin. Abbreviations: CS, chondroitin sulphate; DS, dermatan sulphate; HA, hyaluronate; HS, heparan sulphate.*

stage produced about twice as much CS than DS. At stage HH5 and HH7-9 the relative content of DS and CS was about the same. The concentration of HS increased about 3 fold from stage XIII$^+$ to stages HH4 and HH5 and then increased another 4.5 fold to stage HH7-9. Dermatan sulphate was more than 5 fold predominant to HS from stage XIII$^+$ and through to stage HH5 and 3 fold predominant at stage HH7-9.

Treatment of the same sample of the isolated GAGs with chondroitinase AC II degraded CS and HA to unsaturated-4,5-disaccharides (Δ-disaccharides). The produced disaccharides were analyzed by HPLC which permitted the accurate analysis of crucial parameters such as the pattern of sulphation, types of uronic acid and hexosamine (Table 2). Δdi-OS_{HA} corresponds to Δ-disaccharides produced from HA and Δdi-OScs to those from CS, Δdi-4S and 6S refer to 4- and 6- sulphate produced from CS after digestion with chondroitinase AC II. Table 2 showed that 4- and 6- sulphation of CS increased 14- and 10-fold, respectively, from stage XIII$^+$ to stage HH 7-9. The sulphation pattern of CS had a Δdi-4S: Δdi-6S molar ratio ranging from 4 to 8:1 and Δdi-4S: Δdi-OS molar ratio ranging from 9 to 16:1 and was developmentally regulated. Thus, CS in the early chick embryo was sulphated predominately in the 4-position. Sulphated GAGs were present from the earliest stages, were predominant to the non-sulphated and showed stage-related changes. The presence of both 4- and 6-sulphated disaccharides in CS indicated that both 4 and 6 sulfotransferases were active in the early embryo. The concentration of HA increased more than 2-fold from stage XIII$^+$ to stage HH4, decreased 1.5 times at stage HH5 and was increased again to more than 4-fold from stage HH 5 to stage HH7-9. Hyaluronate could regulate cell behavior through the cell-surface HA receptor proteins CD44 and RHAMM (Reviewed by Sherman *et al.,* 1994) in addition to its space-creating property due to its extensive hydration which could facilitate cellular migration (Solursh, 1976; reviewed by Toole, 1991).

The adhering yolk was removed from the embryos extremely carefully because we had detected DS, smaller amounts of CS and minute amounts of HS, but not HA or heparin, in yolk preparations from fertilized eggs (Fig. 2). The GAGs present did not occur in a free form (Fig. 2, lane 2) but were obtained after treating the yolk precipitates with papain (Fig. 2, lane 1).

Analysis during development of the early embryo indicated a developmental accumulation of HA and sulphated GAGs. Hyaluronate and sulphated GAG synthesis increased markedly at gastrulation when the first major cellular migrations and tissue interactions begin. The sequential increase and decrease in relative concentrations of HA and CS around the time

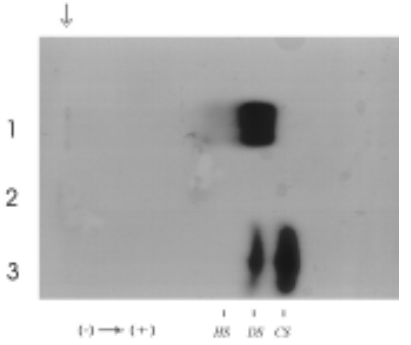

Fig. 2. Separation of glycosaminoglycans (GAGs) isolated from the yolk of fertilized chick eggs. *The GAG fraction from the yolk was obtained by removing lipids and proteins; glycosaminoglycans were separated by cellulose acetate electrophoresis. Lane 1 showed the presence of GAGs after treatment of yolk precipitates with papain. No GAGs were isolated from yolk without papain treatment of the precipitates (lane 2). Identification of GAGs was according to electrophoretic separation of commercial GAG standards (lane 3). Conditions of electrophoresis and abbreviations as indicated in Fig.1.*

of gastrulation was intriguing and it is tempting to think that these differences have morphogenetic significance. Embryonic inductions, being defined as developmentally significant interactions between tissues of dissimilar origin, have been explained in part by differences in the amounts and distribution of extracellular matrix produced by the interacting tissues. The results of this study have provided basic information on the different classes of GAGs that are present in early chick embryos. Such information is essential for an understanding of the role of GAGs and other glycoconjugate chains in early development. Current vibrant research (Bandtlow and Zimmermann, 2000; Esko and Selleck, 2002; Varki, 2002) has focused on the mechanisms that govern proteoglycan diversity, and the mechanisms that regulate the generation of biologically active growth factor/proteoglycan complexes and proteoglycan-dependent induction of transcription factors.

Experimental Procedures

Materials

Twice crystallized papain (EC 3.4.22.2), chondroitinase ABC prepared from *Proteus vulgaris* (EC 4.2.2.4), chondroitinase AC II prepared from *Arthrobacter aurescens* (EC 4.2.2.5), heparin lyase I (EC 4.2.2.7) and heparin lyase III (EC 4.2.2.8) prepared from *Flavobacterium heparinum* and GAG standards and standard preparations of chondroitin disaccharides, Δdi-4S, Δdi-6S and Δdi-0S were purchased from Sigma Chemical Co. (Saint

TABLE 1

CHONDROITIN SULPHATE, DERMATAN SULPHATE AND HEPARAN SULPHATE CONTENT IN EARLY CHICK EMBRYOS

Developmental stage of embryos	*µg GAG / embryo*		
	CS	***DS***	***HS***
XIII$^+$	0.28	0.784	0.152
HH3	1.5	2.25	0.413
HH4	4.56	2.55	0.450
HH5	2.29	2.13	0.467
HH7-9	5.76	6.5	2.16

TABLE 2

IDENTIFICATION OF DISACCHARIDES BY HPLC ANALYSIS AFTER DIGESTION OF GAGs WITH CHONDROITINASE AC II*

Developmental stage of embryos	*Δdi-0S_{HA}*	*Δdi-0S_{CS}*	*Δdi-4S*	*Δdi-6S*	*Δdi-4S/ Δdi-6S*	*Δdi-4S/ Δdi-0S_{CS}*
XIII$^+$	16	24	217	37	5.86	9.04
HH3	12.3	25	254	61	4.16	10.16
HH4	36	30	410	93	4.41	13.67
HH5	23	40	-	-	-	-
HH7-9	96	184	3010	370	8.14	16.36

* The results are expressed as pg of disaccharides per embryo.

Louis, MO, USA). All other chemicals used were of the best commercially available grade.

Embryos

Chick embryos at stage XIII+ (late blastula), stages HH3 (intermediate primitive streak/mid-gastrula), HH4 (definitive primitive streak/gastrula), HH5 (head process/early neurula), and HH7-9 (6 somites) were obtained after incubation of eggs at 37°C. Roman and arabic numerals indicate stage of chick embryo development according to Eyal-Giladi and Kochav (1976) and Hamburger and Hamilton (1951), respectively. Embryos were removed from the eggs and the adhering yolk was cleaned off using fine dissecting needles. The cleaned embryos were used to isolate glycosaminoglycans (GAGs).

Isolation of GAGs

The GAG fraction was obtained after removing lipids and proteins (Svennerholm and Fredman, 1980; Papageorgakopoulou *et al.,* 2001) from early chick embryos and from yolk of fertilized eggs. Ten embryos of stage XIII+, 8 embryos of each stage HH3 and HH4, 7 embryos of stage HH5, 1 embryo of stage HH7-9, and yolk from 1 fertilized egg (the embryo was removed), separately, were homogenised in 3 vol 0.1 M Tris-HCl (pH 7.5-8.0) buffer on ice. The homogenates were mixed with 10 vol methanol/ chloroform (2:1 v/v) for about 4 h, at room temperature under periodic stirring; the mixture was centrifuged for 20 min, at 3500 rpm, room temperature and the supernatant was discarded. The residues were reextracted with methanol/chloroform (2:1 v/v) and after centrifugation the precipitates were washed with ethanol; the supernatant was discarded. The lipid-free precipitate was dried, dissolved in 0.1 M sodium acetate buffer, pH 6.5, containing 20 mM cysteine and 4 mM EDTA (disodium salt) and treated with papain (100 u/sample) twice (Papageorgakopoulou *et al.,* 2001). Papain treatment was performed for two 24 h periods at 60°C in a shaking bath. The macromolecules after the papain treatment were precipitated by the addition of 7 volumes of ethanol at 0°C overnight. The GAG fraction (ethanolic precipitate) was dissolved in 120 μl ddH_2O and the uronic acid was determined by the borate carbazole reaction with glucuronolactone as standard (Bitter and Muir, 1962); the protein content was determined from the ethanolic supernatant by the method of Bradford (1976). From the GAG fraction solution, 5 μl was removed for the separation of GAGs by cellulose acetate electrophoresis and the remaining was used for the chondroitinase treatment.

The GAGs were treated either with chondroitinase ABC to degrade chondroitin sulphate, dermatan sulphate and hyaluronate (Yamagata *et al.,* 1968), or with chondroitinase AC II to degrade chondroitin sulphate and hyaluronate (Yamagata *et al.,* 1968), and with heparin lyases I (0.1u/25 μg UA), and III (0.01u/25 μg UA) to degrade heparan sulphate and heparin (Papageorgakopoulou *et al.,* 2001). The macromolecules resistant to chondroitinases and heparin lyases were precipitated by the addition of 7 vols of ethanol at 0°C overnight and the precipitates were subjected to cellulose acetate electrophoresis (Theocharis *et al.,* 2001). The ethanolic supernatants from chondroitinase ABC and AC were freeze-dried and analysed for their disaccharide content by high pressure liquid chromatography (HPLC) (Karamanos *et al.,* 1995).

Electrophoresis on cellulose acetate membranes was carried out in 0.1 M pyridine – 0.47 M formic acid pH 3.1 for 45 min at 2 mA/cm followed by staining with 0.5% (w/v) toluidine-blue in 15% (v/v) ethanol (Theocharis *et al.,* 2001).

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