Developmental Changes in Serum UDP-GlcA:Chondroitin Glucuronyltransferase Activity*

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Bovine, rat, and chicken UDP-GlcA:chondroitin glucuronyltransferase activities in sera during prenatal and postnatal development were systematically measured with polymeric chondroitin as an exogenous acceptor and with UDP-[3-14C]GlcA as a donor. The results indicated that the activity changed markedly with development in all species examined. Specifically, the activity was the highest at the middle prenatal stage in the bovine and chicken sera and at the late prenatal stage in the rat serum, and it decreased sharply thereafter in all three species. Although the origin of the serum enzyme has not yet been determined, these changes may reflect developmentally regulated biosynthesis of chondroitin sulfate and also suggest that the glucuronyltransferase could be a regulatory enzyme controlling the expression of chondroitin sulfate.

A wide variety of proteoglycans with characteristic sulfated glycosaminoglycan (GAG)
chains exhibit tissue-specific and developmentally regulated synthesis (1) and have been implicated in the regulation and maintenance of cell proliferation, cytodifferentiation, and tissue morphogenesis (2). The structures of cartilage chondroitin sulfate GAGs change with normal embryonic development and growth or aging (3–5). More recently, chondroitin sulfate has been implicated in the development of the rat central nervous system (6, 7). The basis for the developmentally regulated and tissue-specific synthesis of chondroitin sulfate and other GAGs is at present poorly understood. As the GAG structures are largely determined by the specificities of the glycosyltransferases and sulfotransferases responsible for their synthesis, it is presumed that differential expression of these enzymes is most likely the key for the controlled synthesis of GAGs. However, few studies have systematically investigated the degree to which specific glycosyltransferases are differentially expressed.

Chondroitin sulfate is synthesized as a proteoglycan and contains a number of chondroitin sulfate side chains covalently linked to the core protein. Chondroitin sulfate has a linear polymer structure that possesses repetitive, sulfated disaccharide units containing glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) (8, 9). The GlcA-GalNAc disaccharide units are synthesized by the alternating actions of glucuronyl- and N-acetylgalactosaminyltransferases (8).

While searching for glycosyltransferases involved in chondroitin sulfate biosynthesis in various animal tissues, we detected in animal sera the two enzymes UDP-GalNAc:chondroitin N-acetylgalactosaminyltransferase (GalNAc-T) (10) and UDP-GlcA:chondroitin glucuronyltransferase (GlcA-T), which are probably responsible for polymerization of the chondroitin sulfate backbone, and found the development-dependent activity of the latter enzyme. In this report, we present evidence for developmental changes in GlcA-T activity in sera from various animal species.

EXPERIMENTAL PROCEDURES

Materials—UDP-[1-14C]GlcA (285.2 mCi/mmol) was purchased from DuPont NEN. Unlabeled UDP-GlcA was from Sigma. Chondroitin (a chemically desulfated derivative of whale cartilage chondroitin sulfate A), five unsaturated standard disaccharides derived from chondroitin sulfate, Δ4,5GlcAβ1–3GalNAc, Δ4,5GlcAβ1–3GalNAc(4-O-sulfate), Δ4,5GlcAβ1–3GalNAcβ1–3GalNAc(6-O-sulfate), Δ4,5GlcAβ1–3GalNAc, 4-O-sulfate), Δ4,5GlcAβ1–3GalNAc(6-O-sulfate), and Δ4,5GlcAβ1–2-sulfate, β1–3GalNAc(6-O-sulfate), Arthrobacter aureus chondroitinase AC-II (EC 4.2.2.5), and bovine liver β-glucuronidase (EC 3.2.1.31) were purchased from Seikagaku Corp., Tokyo. N-Acetylgalactosamine GlcAβ1–3GalNAc was a gift from Dr. K. Yoshida (Seikagaku Corp.). Sephadex G-25 was obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden. All other reagents and chemicals were of the highest quality available.

Sera—Fetal, newborn calf, calf, and adult bovine sera were purchased from Life Technologies, Inc., Japan, Tokyo. The other sera were prepared from fresh blood obtained as follows. Adult bovine blood was supplied by a local slaughterhouse, and adult human blood was donated by volunteers; newborn human blood taken from a fresh umbilical vein was a gift from Dr. N. Sagawa (Kyoto University); rat and chicken blood was obtained from Wistar rats and White Leghorn chickens, respectively; fetal rat blood and chick embryo blood were obtained with a Pipetman (Gilon) through an opening made in jugular blood vessels and the umbilical vein, respectively, with care to minimize hemolysis; fetal calf plasma was prepared by mixing fresh blood with 30% (w/v) acid citrate dextrose, a generous gift from Dr. M. Okayama (Kyoto Sangyo University). Preparation of serum from fetal plasma was carried out by inducing clot formation with thrombin/CaCl2 (11).

GlcA-T Assay—In a preliminary study, the assay conditions for GlcA-T were established by examining various factors such as buffers, metal ions, substrate concentrations, and inhibitors for UDP-GlcA degradation (the details will be published elsewhere). The assay mixture contained 20 μl of serum, 300 μg of chondroitin, 14.3 μM UDP-[3-14C]GlcA (1.66 × 105 dpm), 50 mM sodium acetate buffer, pH 5.6, and 171 μM ATP in a total volume of 35 μl. Incubations were carried out at 37°C for 4 h, and incorporation of [3-14C]GlcA into chondroitin was determined by the paper disc method (12). Briefly, after 4 h of incubation, the reaction mixtures (35 μl) were spotted onto 2.5-cm Whatman grade 1 paper discs, which were sequentially washed in n-butyc acid, 0.5% ammonia (5/3, v/v), ethanol/ether (2/1, v/v), and ether, and then air-dried. They were finally placed in 0.75 ml of 0.2 M NH4HCO3, and then the radioactivity was counted with a liquid scintillation counter. Net [3-14C]GlcA incorporation into the polymeric chondroitin was calculated by subtraction of the blank value obtained in its absence. Under the established incubation conditions for GlcA-T, GlcA incorporation into polymer chondroitin was proportional to the incubation time and the serum protein up to 6 h and 0.5 mg, respectively.

Characterization of the Reaction Products—Isolation of the products from the GlcA-T reaction was carried out by gel filtration on a Sephadex
G-25 column (1.0 × 105 cm) equilibrated with 0.25 M NH₄HCO₃, 7% 1-propanol. The radioactive peak observed in the void volume contained the product, and it was pooled and evaporated to dryness. Digestion of the isolated product was carried out overnight at 37°C with 100 mIU of β-glucuronidase in a total volume of 50 μl of 0.1% sodium acetate buffer, pH 4.5, or 100 mIU of chondroitinase AC-II in a total volume of 50 μl of 0.1 M sodium acetate buffer, pH 6.0. Each enzyme digest was analyzed using the same Sephadex G-25 column as above. To further identify the reaction product, the resultant radiolabeled materials from the chondroitinase AC-II digestion were isolated by gel filtration and co-chromatographed by HPLC on an amine-bound silica column as described previously (13, 14). The radioactive peak was pooled and analyzed by HPLC on an amine-bound silica column as described previously (13, 14).

RESULTS

Developmental Regulation of the GlcA- and GalNAc-transferase Activities in Bovine Sera—Two enzymes, GlcA-T and GalNAc-T, are believed to be involved in the polymerization of chondroitin sulfate chains. To investigate the relationship between GlcA-T and GalNAc-T activities during development, their activities were measured in fetal bovine, newborn calf, and adult bovine sera with exogenous chondroitin as an acceptor. As shown in Fig. 1, the GlcA-T activity was the highest in fetal bovine sera. Thereafter, the activity gradually decreased with age, and in adult bovine sera little activity was detected. In contrast, the level of the GalNAc-T activity did not change markedly during development as we reported previously (10). Accordingly, further studies were performed with GlcA-T.

Characterization of the GlcA-T Reaction Products—To identify the GlcA-T reaction products, exogenous chondroitin was labeled with [14C]GlcA using fetal bovine serum as an enzyme source, and the products were isolated and then subjected to gel filtration analysis after β-glucuronidase or chondroitinase AC-II treatment. As shown in Fig. 2, the labeled products were completely digested by β-glucuronidase yielding quantitatively a 14C-labeled peak at the position of free [14C]GlcA. These results indicate that GlcA was indeed transferred to polymer chondroitin through a β-linkage. Furthermore, the labeled products were completely digested by chondroitinase AC-II, yielding quantitatively a 14C-labeled peak at the position of disaccharides. The radioactive peak was pooled and analyzed by HPLC on an amine-bound silica column. As shown in Fig. 3, only a single radioactive peak was detected at the elution position of N-acetylchondrosine GlcAβ1-3GalNAc, which was separated from its unsaturated counterpart, GlcAβ1-3GalNAc. These results indicate that GlcA was transferred exclusively to the nonreducing terminal GalNAc of polymer chondroitin, most likely through a β1-3 linkage. The elution position of two other possible disaccharides GlcAβ1-4GalNAc and GlcAβ1-6GalNAc could not be determined due to the lack of the authentic disaccharides.

Developmental Changes of the GlcA-T Activity in Various Animal Sera—To investigate the general validity of the developmental changes in the GlcA-T activity observed above, the GlcA-T activity was measured in sera during bovine, rat, and chicken prenatal and postnatal development. As can be seen in Fig. 4, the GlcA-T activity showed similar developmentally regulated changes in the various sera examined. Generally, the enzyme activity reached a maximum during the prenatal period and then sharply declined with development. The activity
was almost undetectable in adult specimens including human serum. Specifically, the activity was the highest at the middle prenatal stage in the bovine and chicken sera and at the late prenatal stage in the rat sera.

**DISCUSSION**

In this study, development-associated changes in serum GlcA-T activity involving chondroitin sulfate synthesis were demonstrated in various animal species, while the associated GalNAc-T activity did not change markedly during development. These soluble enzymes presumably represent truncated forms of the membrane-bound Golgi enzymes lacking the transmembrane domain, since the biosynthesis of chondroitin sulfate occurs in the Golgi apparatus where such biosynthetic enzymes normally reside. Although it is as yet unknown from which tissues these enzymes are secreted into the bloodstream and what their physiological significance is, it is conceivable that the changes reflect development-associated biosynthesis of chondroitin sulfate in the tissues from which they originate. It has been reported in this regard that the structures of the proteoglycan subunits in calf and human articular cartilage change considerably between the fetal and the mature adult stages, and especially chondroitin sulfate chains become shorter with age (3, 4).

In addition, we have found that cultured chick embryo chondrocytes secrete the GlcA-T into the culture media. Considering the present results that the GlcA-T activ-

postnatal stage (15). It is intriguing to investigate whether these enzymes and the GlcA-T originate from the same tissue and whether their synthesis and/or secretion are controlled in a coupled manner at the transcriptional and/or translational level.

The differential changing patterns observed for the GlcA-T and the GalNAc-T activities (see Fig. 1) are of particular interest. The differences may indicate that these two enzymes are totally different and the mechanism of polymerization of chondroitin sulfate chains is distinct from that of heparin/heparan sulfate chains, which involves a single enzyme, GlcA/GlcNAc transferase (16). Alternatively, the observed decrease in the GlcA-T activity during development may be associated with increasing concentrations of an unidentified inhibitor for the enzyme. Conclusive proof that the GlcA-T and the GalNAc-T are two separate enzymes will require purification and characterization of the corresponding enzymes.

The carbohydrate moieties of glycoconjugates on the surfaces of cells are known to undergo various changes during the malignant transformation of cells. Many of these carbohydrate structures are described as oncofetal antigens because they are most abundant in early fetal development, and their expression is developmentally regulated (17, 18). Moreover, the antigens and sometimes the glycosyltransferases responsible for their synthesis are actively shed into the blood from cancer cells and therefore can be detected and measured in cancer patients’ sera as tumor markers (17). In view of the observation that the GlcA-T is secreted into the bloodstream in a developmentally associated manner, evaluation of GlcA-T as a new tumor marker and screening tests for a possible tumor-related increase in the GlcA-T activity in sera from cancer patients would be of interest. In fact, appreciable enzyme activity was observed in newborn human sera whereas little activity was detected in normal adult sera (see Fig. 4). Furthermore, in preliminary experiments some human cancer cell lines including a melanoma cell line G361 (ATCC CRL-1424), an amelano
tic melanoma cell line C32 (ATCC CRL-1585), and a colon adenocarcinoma cell line LoVo (ATCC CCL-229) were found to abundantly secrete the GlcA-T into their culture media, suggesting that the above may indeed be the case.

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**Fig. 4. Developmental changes in GlcA transferase activity in rat, bovine, chick, and human sera.** The enzyme activity was determined for sera prepared from individual animals and their fetuses as described under “Experimental Procedures.”

### Table 1

| Age                      | Number of Individuals | Glucuronyltransferase Activity |
|--------------------------|-----------------------|-------------------------------|
| 0.5 day fetal            | 5                     | 0.0                            |
| 1-11 day fetal           | 5                     | 0.0                            |
| 16-15 day fetal          | 4                     | 0.5                            |
| 17-16 day fetal          | 4                     | 1.0                            |
| Newborn                  | 4                     | 2.0                            |
| 2 weeks old              | 3                     | 3.0                            |
| 3 weeks old              | 4                     | 4.0                            |
| 4 weeks old              | 4                     | 5.0                            |
| 15 months old            | 2                     | 6.0                            |
| 19 day old               | 4                     | 7.0                            |
| 17-19 day old            | 4                     | 8.0                            |
| Newborn                  | 4                     | 9.0                            |
| 7 weeks old              | 4                     | 10.0                           |
| 12 weeks old             | 4                     | 11.0                           |
| 20 weeks old             | 3                     | 12.0                           |
| 25 days old              | 2                     | 13.0                           |
| 5 months old             | 2                     | 14.0                           |
| 9 months old             | 3                     | 15.0                           |
| 12 months old            | 3                     | 16.0                           |
| 20 months old            | 3                     | 17.0                           |
| 25 months old            | 2                     | 18.0                           |
| 30 months old            | 2                     | 19.0                           |
| Adult                    | 2                     | 20.0                           |

2. H. Kitagawa, M. Ujikawa, and K. Sugahara, unpublished data.
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