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Distribution of Specific Glycoconjugates in Early Mouse Embryonic Notochord and Paraxial Mesenchyme

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ABSTRACT

It is well known that glycoconjugate components of the cell surface and extracellular matrix, play an essential role(s) in many developmental phenomena such as cell differentiation, migration, and cellular interactions. The purpose of this study was to investigate distribution of this macromolecules during differentiation of the notochord and paraxial mesoderm. Formalin fixed paraffin sections of 9 to 16 days of BALB/c mouse embryos were processed for histochemical studies using four different horseradish peroxidase conjugated lectins including wheat germ agglutinin (WGA), Griffonia simplicifolia (GSA1-B4), Arachis hypogaea (PNA) and Lotus tetragonolobus (LTA), specific for sialic acid, galactose, N-acetylgalactosamine (GalNAc) and fucose terminal sugar, respectively. Our results showed that in primordial of the developing vertebrae PNA sensitive glycoconjugate appeared on gestational day 13 and increased to gestational day 15 significantly (*P*<0.05) and disappeared later. GSA1-B4 revealed no reaction and LTA and WGA reactions were observed only in notochord. Among the lectins that were used in this study, only PNA showed continuous and strong reaction just during gestational day 13 to 15. This finding suggests that PNA temporally regulated changes occurred during early vertebral development in mouse embryos and probably their specific terminal sugar plays a key role(s) during prechondrogenic vertebrae. *Iran. Biomed. J. 9 (1): 21-26, 2005*

Keywords: Notochord, Glycoconjugate, Mesenchyme, Lectin histochemistry

INTRODUCTION

Formation of the notochord is one of the earliest and most obvious events of axis development in vertebrate embryos. Early investigations in birds and mammals show that prospective notochordal cells arise from Hensen's node. They lie beneath the middle of neural plate and induce it. Notochord retains a close spatial relationship with developing neuroepithelium throughout the period of neurulation [1]. In addition to the supporting role, notochord is also known to have a role in the developmental patterning of midline and paraxial structures, for example, notochord has the ability to induce cell fate changes such as differentiation of floor plate [2], motor neurons [3], oligodendrocytes [4], sclerotome [5], somites [6], dorsal pancreatic bud and pancreatic islets [7] and axial vessels [8]. Molecules involved in these induction processes are beginning to be characterized, including hedgehog signaling pathway [9, 2-3, 5].

The paraxial mesoderm, which lies adjacent to the neural tube and notochord, segments into transient epithelial blocks termed somites. As somitic cells differentiate, the somites are divided into a ventral mesenchymal compartment, the sclerotome, and dorsal epithelial compartment, the dermomyotome. While dermomyotomal cells give rise to the dermis and skeletal muscles [10, 5-6], the sclerotomal cells delaminate and give rise to vertebrae, ribs and intervertebral discs.

Several lines of evidence indicate that the patterning of dorsoventral aspects of the somite is the result of a combination of inductive signals drived from the neighboring structures. Signals from the notochord and floor plate ventralize the somite and induce sclerotome formation and

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ensuing chondrification of these cells [5-6], and repress dermomyotome formation [11], while signals from the dorsal neural tube and surface ectoderm seem to counteract the activity of the ventralizing signals and induce dermomyotome formation [12, 10].

Despite of extensive morphological and genetic studies of notochord and parsaxial mesenchyme development, few studies have examined the distribution of glycoconjugates on these structures. These materials at the cell surface or within the intercellular matrix take part in a wide range of biological functions, such as cell-cell contact, cell proliferation, migration, differenti-ation, as well as cellular adhesion [13]. Many of these carbohydrate moieties change during cellular development and differentiation [13]. The glycosylation pattern of cells and tissues give insights into spatially and temporally regulated developmental process that can be detected histochemically using plant lectins with specific affinities for sugar moieties [14].

Lectins are naturally polypeptides that bind specifically to carbohydrate (sugars) residues of glycoconjugates and have extensively been used as probes in studies of cell surface interaction, and other developmental phenomena in several different developing systems, including neural tube [15].

The purpose of the present study was to investigate the charactristics, distribution and developmental changes of certain glycoconjugates in early development of notochord and paraxial mesenchyme by means of lectin histochemistry.

MATERIALS AND METHODS

Preparation of mouse embryos. Twenty female BALB/c mice were individually caged and fed by standard laboratory chow and water ad libitium under controlled conditions (12:12 light/dark, temperature 24 ± 1˚C). They were mated overnight with ten fertile males of the same strain and subsequently examined in the next morning for the presence of the vaginal plug, which was considered as day 0 of gestation (GD0). From gestational day (GD) 9 to 16, animals were cesareaned under deep anesthesia by inhalation of chloroform (two mice for every GD). Embryos were dissected and freed carefully of their extraembryonic membranes (mean numbers of embryos were eight for each pregnant mouse).

Preparation of tissue sections. For each GD, four embryos were washed in normal saline, and fixed in 10% formaldehyde overnight, at room temperature. Tissue blocks were dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Transverse serial sections with six-micrometer thickness were provided by rotary microtome.

Lectin histochemistry. Four HRP-conjugated lectins were purchased from Sigma chemical company (USA) such as Griffonia (Bandeiraea) simplicifolia1-B4 (GSA1-B4), Arachis hypogaea (peanut) (PNA), Lotus tetragonolobus (asparagus pea) (LTA) and *Triticum vulgaris* (wheat germ agglutinin) (WGA) specific for D- Gal, D-Gal-(β1→3)-D-Gal Nac, α- L-fucose and sialic acid, respectively. Lectins were diluted with PBS 10-20 µg in 0.1 M PBS [16].

Five sections of thoracic region of each embryo were chosen randomly and deparaffinized in xylene and then hydrated and rinsed for 10 minutes in 0.1 M PBS (pH 7.4). Intrinsic peroxidase activity was blocked with hydrogen peroxide-methanol 1:100 for 45 minutes in the dark [14]. All sections were incubated with lectins, at room temperature for 2 hours. After extensive rinising in PBS, they were incubated in diaminobenzidine (DAB)-hydrogen peroxidase substrate medium (pH 7.0) at room temperature for 15 minutes. All sections were counterstained with a 1% solution of Alcian blue at pH 2.5 for five minutes [16]. Finally, the sections were dehydrated in graded ethanol and cleared in xylene and mounted.

The affinities of the tested lectins were viewed by three examiners separately under light microscope (Olympus AH-2) and grading was done according to Gong method from zero to three that is considered positive [17]. Thereafter, comparisons were made among these lectins and also across the different types of tissues and developmental stages. Data analyses by none parametric Kruskal Wallis test and differences less than 0.05 were considered as statistically significant.

RESULTS

Notochord and paraxial mesenchyme develop in a craniocaudal direction with time advancing and their histochemical changes can be investigated by comparing similar sections in different stages of development. Reactions of glycoconjugates about thoracic sections of different GD have been summarized in Table 1.

No reaction of GSA1-B4 was observed in the notochord and paraxial mesenchyme in all examined specimens (Fig. 1).

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Table 1. Binding of lectins in the notochord and the paraxial mesenchyme during 9-16 days of pregnancy.

Lectin tested		Gestational day							
		E9	E10	E11	E12	E13	E14	E15	E16
GSA1-B4	NC	-	-	-	-	-	-	-	-
	PM	-	-	-	-	-	-	-	-
LTA	NC	+	++	++	+	-	-	-	-
	PM	-	-	-	-	-	-	-	-
PNA	NC	+	++	++	+	-	-	-	-
	PM	+	+	+	+	++	+++	+++	-
WGA	NC	++	+++	+++	+++	++	+	+	+
	PM	+	+	+	+	+	+	+	+

Staining intensity based on estimated scale from 0 to +++, with: negative reaction = (-); weak = (+); moderate = (++); severe = (+++); NC = notochordal cells; PM = paraxial mesenchyme; E= embryonic day; GSA1-B4, Griffonia simplicifolia1-B4; LTA, Lotus tetragonolobus; PNA, peanut agglutinin; WGA, wheat germ agglutinin.

A weak reaction of LTA was observed in notochordal cells at the beginning of GD9. This reaction increased on GD10 ($P<0.05$) (Fig. 2) and continued on GD11. But this staining intensity decreased from GD12 ($P<0.05$) and finally disappeared. Paraxial mesenchyme showed no reaction at any tested section (Fig. 2).

From GD9 to GD12, weak to moderate reaction of PNA was observed in the notochord and the paraxial mesenchyme as well. This reaction became negative in notochord thereafter at the beginning of GD13, significant changes appeared ($P<0.05$) and moderate staining was observed in the mesenchymal cells (Fig. 3) and increased on GD14 ($P<0.05$) (Fig. 4). This reaction on GD15 was restricted only to outer zone of future centrum and forming laminae of vertebrae (Fig. 5), and disappeared in chondroblasts around the notochord. On GD16, the reaction was observed only in forming laminae.

A week to moderate and uniform staining of WGA was found in paraxial mesenchymal cells during all GD investigated, but notochordal cells showed an intense reaction from GD10 to GD12 (Fig. 6) and decreased thereafter ($P<0.05$).

DISCUSSION

Notochord is a mesodermal derivative in vertebrates and is the most important organizer in the region of the axial mesenchyme, which influences morphogenetic process in the sclerotome, differentiation of the neural tube and dorsal pancreatic bud [2-7].

In the present study, extensive glycosylation changes in the notochordal tissue were observed. These changes did not constrict to the cytoplasm of the cells, but were observed at plasma membranes and intercellular matrix. The notochord binds to most of the lectins that indicate the presence of sugar structures most probably related to proteoglycan, adhesion molecules or secreted glycoproteins synthesized by these cells [14]. Hoedt-Schmidt *et al.* [18] have reported that O-linked GalNAc-containing oligosaccharides, which are parts of chondroitin and dermatan sulphate molecules, are PNA positive. Moreover, chondroitin or keratan sulphate-rich proteoglycans has/have been found in embryonic chicken and human notochord [19].

Lectin reactivity of notochordal cell surface especially for PNA may reflect binding to glycoconjugates that might be adhesion molecules [20], for example cadherins (adhesion molecules at desmosomal junction) have been detected on human notochordal cells [14]. Griffith and Sanders [15] have reported that PNA-binding structures are oligosaccharides which may be important for cellular interactions during morphogenesis process. They found that PNA can be a characteristic marker for mesodermal tissues in the chick embryo.

Glycoconjugates detected by lectins may also belong to the secreted glycoproteins of the notochord such as sonic hedgehog, which functions as a signaling developmental molecule [21- 23, 3, 5-7, 16]. In vertebrate, sonic hedgehog secrets from the notochord and specifies the floor plate at high-level activities, dorsoventral patterning of somites and left-right asymmetry [3]. Kim *et al.* [22] have reported that sonic hedgehog is a secreted glycoprotein which is crucial to normal embryonic development. This substance is released from notochord and acts in concentration-dependent manner. Mortella *et al.* [23] have shown that in abnormal notochord, disturbance of secreted sonic hedgehog is responsible for possible mechanism in etiology of foregut anomalies.

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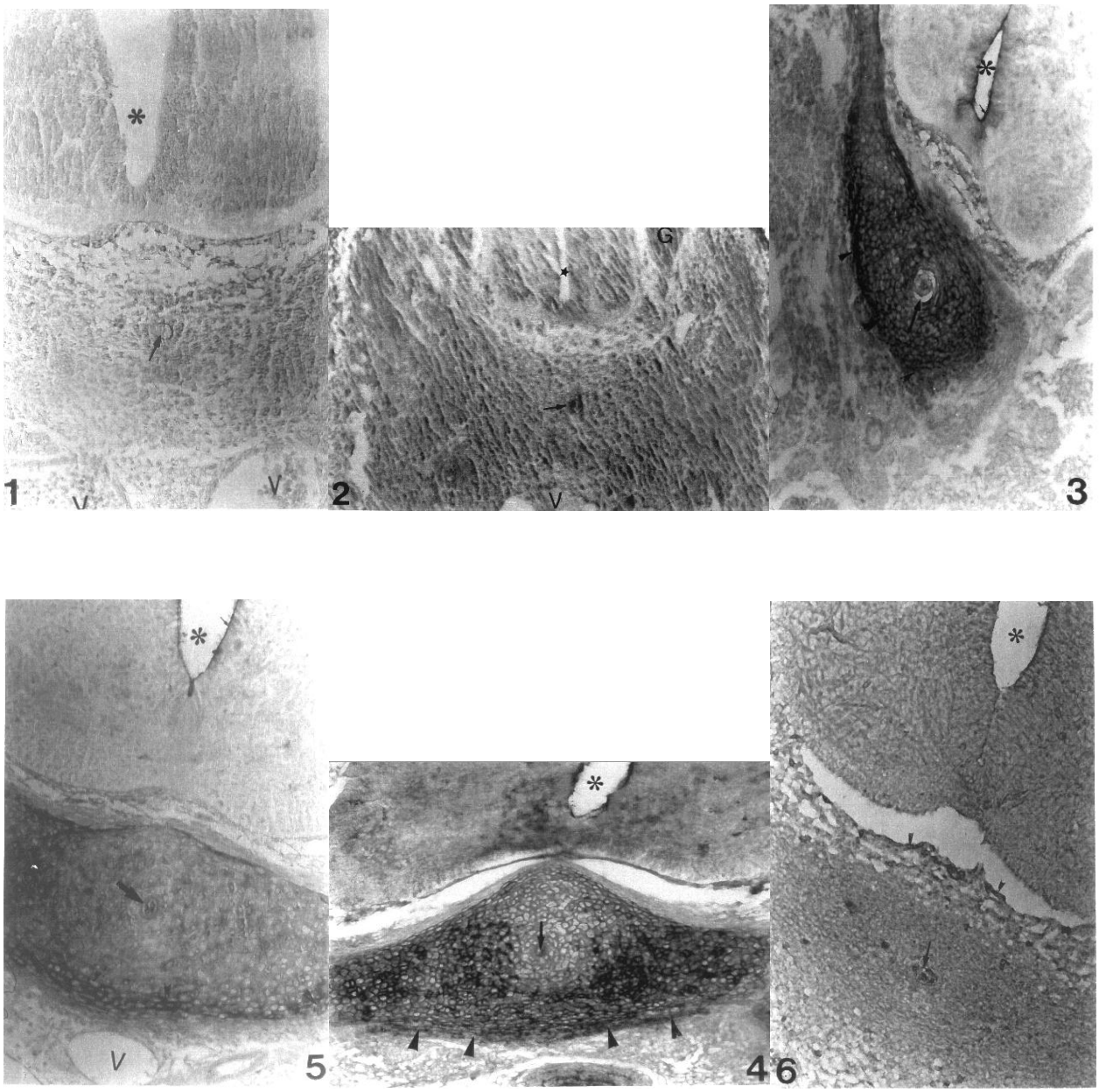

Fig. 1. **(1)**, Photomicrograph of day 11 mouse embryo, cross section of notochord, paraxial mesenchyme and ventral portion of neural tube; incubated with GSA1-B4. Notochord (arrow), paraxial mesenchyme and neural tube (Asterisk) show no reaction. V= vessel. Magnification, ×200; **(2)**, Photomicrograph of day 10 mouse embryo, cross section of notochord, paraxial mesenchyme and ventral portion of neural tube; incubated with LTA. Notochord (arrow) shows moderate reaction. Notochordal sheath reacted with Alcian blue. Neural tube (Star), spinal ganglion (G), V= vessel. Magnification ×200; **(3)**, 3-Photomicrograph of day 13 mouse embryo, cross section of notochord, paraxial mesenchyme and ventral portion of neural tube, incubated with PNA. Degenerating notochord (arrow) shows no reaction. Paraxial mesenchyme shows intense reaction especially at outer zone of future centrum (arrowheads). Staining of luminal surface (small arrow) of neural tube (Asterisk) is severe. Magnification, ×200; **(4)**, Photomicrograph of day 14 mouse embryo, cross section of notochord, paraxial mesenchyme and ventral portion of neural tube, incubated with PNA. Degenerating notochord (arrow) shows no reaction. Paraxial mesenchyme shows intense reaction especially at outer zone of future centrum (arrowheads). This staining is disappearing around the notochord. Asterisk shows neural tube. Magnification, ×200; and in **(5)**, Paraxial mesenchyme reaction is also negative except at outer most zone of future centrum (arrowheads). Reacted parts (small arrow) of neural tube (Asterisk) is severe. V= vessel. Magnification, ×200; **(6)**, Photomicrograph of day 12 mouse embryo, cross section of notochord, paraxial mesenchyme and ventral portion of neural tube, incubated with WGA. Notochord (arrow) shows intense reaction. Neural tube (Asterisk) and paraxial mesenchyme show weak to moderate and uniform reaction. This reaction at luminal surface of neural tube is severe. Some meningeal cells (arrowheads) also stained intensely. Magnification, ×200.

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Glycoconjugates that bind to PNA lectin may be cell surface receptors, carbohydrates of proteoglycans, or glycoprotein like fibronectin or tenascin [24, 25]. Fibronectin and tenascin collaborate in regulating collagenase gene expression in fibroblast in culture and this may be an important feature for regulation of cell adhesion, cell migration and morphogenesis [25]. According to Gotz *et al.* [19], the axial mesenchyme of the early human embryo is rich in sulphated glycosaminoglycans and hyaloronic acid and contains glycoproteins like fibronectin, tenascin and laminin, but no keratan sulphate. Hemming and Saxod, [26] demonsrated that keratan sulphate proteoglycans are not binding molecules for PNA. According to the barrier theory, the axial mesenchyme of vertebrates belongs to a sort of tissue which inhibits the ingrowth of nerve cones and neural crest cells [27]. These tissues show PNA binding [24]. Indeed the unsegmented axial mesenchyme around the notochord lacks nerves, invading neural crest cells and also blood vessels due to the inhibiting effects of the notochord and notochord-drived proteoglycans [19]. Up to now, it has not been defined which PNA-binding molecules are responsible for this inhibition [27]. Landolt *et al.* [28] reported that versican proteoglycan is selectively expressed in embryonic tissues that act as barriers to neural crest cell migration and sensory and motor axonal outgrowth.

In this study, weak WGA reaction was found in paraxial mesenchyme, therefore, only few sialic acids are present in this region [29]. Severe notochordal reaction by this lectin indicates the presence of sialic acid structure that increases from G10 to G12 and decreases at later stages of development. The absence or weak reaction of this lectin binding in axial mesenchyme in the present study could be related to the lack of migration, since axial mesenchymal cells no longer migrate in this area [14].

Fucose-binding LTA showed weak to moderate reaction only in notochordal cells from GD9 to GD12 and no reaction in mesenchymal cells. It has been reported that this lectin has shown reactivity in both notochord and floor plate of the neural tube, where sonic hedgehog is present [30].

Negative reaction of galactose sensitive lectin, GSA1-B4, in all investigated embryonic days revealed that this terminal sugar has no role in development of notochord and axial mesenchyme, at least in BALB/c mice.

PNA reaction and its changes revealed that glycoconjugates with terminal (Gal β1→3 GalNAc) groups are present in the prechondro-genic vertebral body anlagen and later in the vertebral arch formation, and probably contribute to the conversion of mesenchymal cells into the chondroblasts during the development of vertebrae [14]. However, species differences are a well-known phenomenon in lectin histochemistry; for this reason, in the human embryo, mesenchymal areas may exhibit altered glycosylation pattern as found and discussed by Gotz and Quondumatteo [14].

In conclusion, our findings indicate that temporally regulated changes do not occur in glycosylation of mouse embryos during early vertebral development except for PNA. However, the binding of other lectins, used in this study, shows their contribution to the biological functions of notochord and mesenchyme in phenomena such as preservation of tissue structure, proliferation, differentiation and preparation of the axial region for subsequent processes.

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