

Distribution of some Glycoconjugates in the Notochord and Developing Gut during Early Morphogenesis in Balb/c Mouse Embryos

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Abstract

Background: Embryonic endoderm germinal layer, affected by notochord inductions, forms the primary gut epithelium and parenchyma of its derived organs. This study aims to determine some expressed glycoconjugates and their potential function in notochord and developing gut.

Materials and Methods : In this descriptive-analytical study, 9 and 10 embryonic days (ED) of Balb/c mouse embryos were fixed in formalin and microscopic sections were prepared from them. These sections were processed for histochemical studies and then they were incubated with 6 different HRP conjugated lectins, including VVA, SBA, and PNA specific to identify terminal sugar (N-acetylgalactosamine) (GalNac) and lectins of GSA1-B4, LTA and WGA were respectively to identify the terminal sugars of galactose, fructose and sialic acid.

Results: The study results showed that the reactions of notochord and developing gut to VVA lectin were moderate on the 9ED and on the 10ED, they showed a significant difference ($p < 0.001$) from the day before and were severely assessed. Other GalNac specific lectins react severely and almost similarly to notochord and developing gut on the studied days. The other lectins in these two organs did not react similarly.

Conclusion: According to the findings of this study, it seems that glycoconjugates with GalNac-terminal sugar probably have played a key role in differentiations of notochord and developing gut and may be involved in the interactions between these two organs.

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Introduction

Notochord is an axial structure derived of embryonic mesoderm which appears early in the embryonic development process and in addition of supporting role it has inducing abilities to all nearby tissues and has an essential role in their differentiation such as neural tube, somites, axial vessels and endoderm [1-3]. According to the crucial structural and inductive roles of notochord, this organ has been extensively studied so far in terms of the formation, and morphological and genetic changes, but it has been less considered from molecular aspects especially the study of glycoconjugates and their potential role in the changes of notochord and its inductive interventions. Hence, there is a need for broader research in this respect [4].

Glycoconjugates are macromolecules which are found on the cell surface and extracellular matrix in the form of glycoprotein, glycolipid and proteoglycan. These molecules appear at a certain stage and will be omitted in different ways after doing their developmental task [4, 5]. It is also proven that carbohydrate part of these compounds, especially their terminal sugar, will change parallel to the differentiation process and cellular development.

They are involved in various processes such as cell adhesion, growth, proliferation, differentiation, migration and apoptosis [6-8]. These compounds are important in that they can play a decisive role in the pathogenesis of some congenital diseases [9] in addition to being involved in the process of normal embryonic development and are detectable through Lectin Histochemistry technique and have been already studied in the development of so many organs such as eyes [10, 11], ears [12], vertebral column [13], intestines [14], testes [15], and prostate [16]. The embryonic endoderm germinal layer forms the primary gut epithelium as a result of two crown-rump and lateral folding, which includes the foregut, midgut and hindgut. Other layers of intestinal wall are made of visceral mesoderm. Some epithelial buds also form the organs such as liver and pancreas as a result of proliferation and differentiation [2, 3]. The differentiation of endoderm layer and the pancreas formation have a strong dependence on notochord so that the removal of notochord causes intestinal anomalies and avoids formation of the dorsal pancreatic bud [2, 3, 17]. Evidence indicates that the inductive effects of notochord on gut are related to the

time when there is a direct contact between notochord and endoderm [17]. The anomalies resulting from developmental defects of notochord, non-separation of notochord from the primary intestine as well as the excessive growth of notochord has caused some abnormalities in the organs derived from the foregut and hindgut, such as dual-branch pharynx, esophageal cysts, gastric cysts, rectovesical fistula and rectum agenesis [18]. Other observations have shown that the experimental creation of esophageal atresia is associated with notochord anomalies [18].

In some other studies conducted on rats, esophageal atresia and tracheoesophageal fistula are known for the prolonged contact of notochord with the foregut [18]. Given that the differentiations of endoderm embryonic layer and the development of its derived organs, are dependent on notochord inductions, and considering that notochord inductions on this organ are related to the time when notochord and endoderm germinal layer are adjacent to each other [17], and on the other hand, according to the interventions of glycoconjugates in embryonic differentiations, thus, the expression of some glycoconjugates and their changes in notochord and developing gut as well as their possible involvement in the interactions between these two organs in the early morphogenetic period have been studied and reviewed through lectin histochemical technique.

Materials and Methods

Preparation of mouse embryos: In order to conduct this descriptive-analytical study, 40 two-month-old virgin female Balb/c mice were used. They were purchased from Razi (Mashhad) Vaccine and Serum Research Institute. Then, 20 male mice of the same strain were made to intercourse in mating cages. By observing the vaginal plaque, the zero day of pregnancy was determined for each of them and they were kept under the standard conditions of animal house (12 hours of light /12 hours of dark, temperature of 24 ± 1 C and relative humidity of 50%). In the ninth and tenth days of pregnancy, for each stage, first six pregnant mice were deeply anesthetized with ether and then delivered by cesarean section. Thereafter, uterine branches were carefully separated and transferred to the physiological saline in order to isolate membranes. After washing with saline solution, the collected embryos were transferred to the 10% fixative formalin solutions and B4G (6% mercuric chloride, 1% sodium acetate and 1% glutaraldehyde) at laboratory temperature [1, 19-21].

Preparation of tissue sections: After completion of embryo fixation stage, which was performed according to the conventional histological methods, the samples were passaged by passing through ethanol with an increasing concentration and then, after getting dehydrated, they were clarified in xylene. Then, paraffin blocks were prepared from these samples and serial sections of 6 micron thickness

were prepared in horizontal and sagittal directions using the rotary microtome in the histotechnic laboratory.

Lectin histochemical studies: In order to do such studies, 36 sections (for each lectin 6 slices) were randomly selected from each embryonic stage and then, they were relieved of paraffin and hydrated through the regular histochemical method. Then the fixed samples with B4G were removed of deposit [9]. In order to remove endogenous peroxidases, the sections were put in the dark in 1% hydrogen peroxide solution in methanol for 45 minutes [4]. Thereafter, the lectins in table 1 were used, which were purchased from Sigma Company in the conjugated form with HRP (Horse Radish Peroxidase). For this purpose, the mentioned lectins were first diluted with the help of Phosphate Buffered Saline (PBS), so that there would be 10 micrograms of lectin in each milliliter of the resulting solution [1, 19-21].

At the next stage, the sections were incubated with each of the above diluted lectins for two hours. After this stage, tissue samples were washed with buffer and were placed in 0.03% DAB solution (Diamino Benzidine) in phosphate buffer for 5 min and for each 10 ml of the above solution, 20 μ l of hydrogen peroxide was added to the medium [1, 19-21].

Table 1: Different types of lectins and their specific detecting terminal sugars, which have been used in this study

| Lectin Tested | Abbreviation | Carbohydrate-binding specificity |
|---|--------------|--|
| (Vicia villosa Agglutinin) Hairy winter vetch | VVA | Gal 1 3 GalNac > Gal |
| (Soybean agglutinin) Glycine max | SBA | , - D-GalNac > D-Gal |
| (Peanut agglutinin) Arachis hypogaea | PNA | D- Gal (1 3) – D-GalNac |
| (Griffonia simplicifolia agglutinin) | GSA1-B4 | -D-Gal |
| (Lotus tetragonolobus agglutinin) Asparagus pea | LTA | Fuc 1 6GlcNac > Fuc 1 2 Gal 4 [Fuc 3] GlcNac |
| (Wheat germ agglutinin) Triticum vulgare | WGA | [(1 4)-D-GlcNac] 2 or 3 Sialic acid |

Fuc = Fucose, Gal = Galactose, GalNac = N-ethylgalactosamine, Glc = Glucose

At the next stage, all sections, having been washed with water, were put in Alcian Blue solution of pH = 2.5 for 5 minutes to create the background color. In this case, reaction of lectins with the respective terminal sugar was shown with brown color in microscopic observations. In order to observe the performed reactions, the samples were studied and photographed with microscope of Olympus/AH-2. Followed by that, the samples were blindly and separately ranked by a three-member team from zero (no reaction) to four positive (very severe reaction) in accordance with previous experiences [1, 19-21], and based on the severity of reaction with used lectins. Then the mean of the proposed grades for each sample

was determined and the obtained data were compared through nonparametric Kruskal-Wallis test using SPSS-11.5 software, and $p < 0.05$ was considered significant. In order to conduct control tests, some non-conjugated sections were placed adjacent to HRP for 2 hours and were processed like test samples.

Results

Reviewing the results of this research, first histomorphologic changes of notochord and then the expressed glycoconjugates (based on their terminal sugar) in notochord and the gut development in cells and extracellular matrix on the ninth and tenth embryonic days were studied with help of the specific lectins, which are summarized in table 2.

Table 2: Summary of reactions of the tissues studied by tested lectins on the ninth and tenth embryonic days

| Tested Lectins | Notochord | | Developing gut | |
|----------------|-----------|------|----------------|------|
| | 9ED | 10ED | 9ED | 10ED |
| VVA | ++ | +++ | ++ | +++ |
| PNA | +++ | +++ | +++ | +++ |
| SBA | +++ | +++ | +++ | +++ |
| LTA | ++ | ++ | - | - |
| GSA1-B4 | - | - | - | - |
| WGA | ++ | +++ | + | + |

No reaction = (-), mild reaction = (+), moderate reaction = (++) , severe reaction = (+++) and very severe reaction = (++++)
ED = Embryonic Days

Histological findings: The findings show that on the 9th ED, notochord will appear as a process with a channel in its center. In this situation, notochord is surrounded by basement membrane, while it is connected to the neural tube in its dorsal part, and to the developing primary gut in the ventral part (Fig 1 & 2). On the 10th ED, notochord has found a plate form (termed notochordal plate) and has been isolated from the gut, while it is still attached to the neural tube. Notochord sheath has also found a higher resolution. Sclerotomal cells are seen migrating around notochord (Fig 3 & 4).

Lectin histochemical findings: On the 9th ED; notochord and its surrounding sheath showed a relatively moderate reaction to VVA lectin. The reaction of the developing gut to this lectin was also assessed as moderate, while these reactions appeared more severely in the apical region of gut epithelial cells (Fig 1 & 2).

On the 10th ED; the reaction level of notochord and its surrounding sheath as well as primary gut to the VVA lectin increased and showed a significant difference from the previous day ($p < 0.001$) and were severely assessed. The reactions of notochord and its surrounding sheath as well as primary gut to PNA lectin on the 9th ED appeared severely, and continued.



Figure 1: Photomicrograph of cross section of 9 ED, incubated with VVA lectin. In this stage the notochordal process (small star) attached to neural tube (big star) posteriorly and to primary gut (G) anteriorly. Moderate reaction observed to this lectin, while this reaction is more severe in apical region of epithelial cells. Thick arrows show amniotic sac, DA= dorsal aorta, ch= chorion, IEC= intera embryonic coelum. (magnification= $\times 200$)



Figure 2: The same figure number 1 which is shown with higher magnification (magnification = $\times 400$)

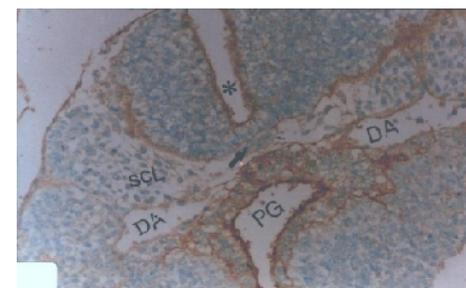


Figure 3: Photomicrograph of cross section of 10 ED, incubated with SBA lectin. In this stage the notochordal process has changed to notochordal plate (thick arrow) which attached to neural tube (big star) posteriorly, while is distinct from primary gut (PG). severe reaction observed to this lectin, while this reaction is more severe in apical region of epithelial cells. DA = dorsal aorta, SCL = sclerotomal cells. (magnification = $\times 100$)

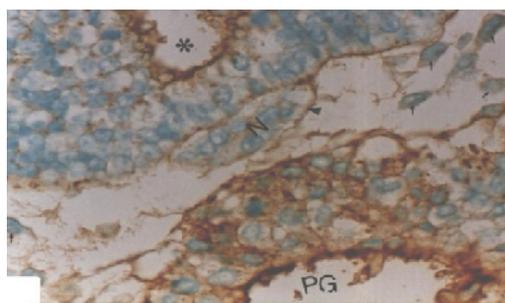


Figure 4: The same figure number 3 with higher magnification N = notochordal plate, big star shows neural tube and head of arrow shows notochordal sheath, P = primitive gut (magnification = $\times 400$)

On the 10th ED with the same severity and no significant difference was observed in these two days in terms of reaction severity. The reaction in the apical region of epithelial cells appeared more severely. SBA lectin also showed a similar reaction like PNA lectin on the 9th and 10th EDs in notochord and the primary gut and no significant difference was seen in the observed reactions; while the reaction severity in the apical region of gut epithelial cells appeared more severely (Fig 3 & 4).

LTA lectin showed only a modest reaction with notochord in both studied days and no significant difference was observed in the reactions to this lectin in the two studied days. GSA1-B4 lectin showed no reaction with the intended tissues. The reaction of WGA lectin with notochord on the ninth day was assessed as moderate. This reaction increased on the tenth embryonic day and was assessed as severe. The difference between the two studied days was statistically significant on the reaction of notochord to this lectin ($p < 0.001$). The reaction of this lectin with developing gut was observed as a mild and in general form in all other tissues.

Discussion

Positive and similar reactions of notochord and developing gut with sensitive lectins to N-acetylgalactosamine (GalNac) terminal sugar indicate that this terminal sugar is probably involved in the differentiation of these two organs and between their interactions. In previous studies, expression and changes of some glycoconjugates have been studied in notochord and its induced tissues such as neural tube [20], somites [1] and axial vessels [21]. In this study, the presence and changes of these compounds have been studied in notochord and another type of its induced tissues, i.e. the endoderm forming the primary gut.

In several previous studies, the role and involvement of notochord in induction of endoderm germinal layer is well shown. In their research, Cleaver et al have shown that differentiation and forming of endoderm layer is dependent on notochord inductions [22]. Some other studies have shown that through secreting Shh molecule, notochord plays an essential role in posterior-anterior pattern formation of somites and the asymmetric formation of left and right organs of body such as heart and intestines [23]. In this regard, Kim et al have reported that Shh glycoprotein secreted from notochord has a critical role in normal embryonic development [17]. In another research, Mortella, et al has shown that in the abnormal notochord, secretion of Shh has been disturbed and this disruption can cause the anomalies of foregut such as tracheoesophageal fistula, esophageal atresia etc [18]. In their studies, Kim, et al have considered the notochord inductions on endoderm necessary to develop pancreas from this embryonic layer [17]. Dilorio, et al have considered these inductions to be

related to the Shh glycoprotein molecule [24]. In the present study, which was conducted to determine the presence and changes of some glycoconjugates in notochord and developing gut, we observed that during the studied days, notochord and developing gut showed a similar reaction with all N-acetylgalactosamine-sensitive lectins used in this study. The reactions were mainly observed in the plasma membrane of these cells and their extracellular matrix. These findings confirm that the GalNac terminal sugar with the spatial arrangement of Gal 1-3-GalNac which are identified by VVA and SBA lectins, and the arrangement of D-Gal (1-3)-D-GalNac which reacts with PNA lectin, are simultaneously expressed in these two organs. These terminal sugars are probably involved in differentiation of these organs as well as between their interactions. In the research conducted by Gotz, et al on human embryos, notochord and endoderm, forming primary gut epithelium, reacted similarly to the used lectins [4].

These researchers have attributed the similarity of the glycoconjugate expressions in these two organs to their temporary merger together and their interactions in the early morphogenetic period [4], which are consistent with our findings.

Positive reaction of notochord with PNA lectin has been shown in various studies and these researchers have attributed these reactions the molecules with adhesive role, oligosaccharides involved in cellular interactions during the morphogenesis process [25], proteoglycans such as chondroitin sulfate and dermatan sulfate [26], cadherin molecules in desmosomal joints [4], glycoproteins on the cell surface with receptor role and other glycoproteins such as fibronectin and tenascin [27]. LTA lectin that is bonded to the -L-Fucose terminal sugar, reacted with notochord cells only moderately, while gut reaction to this lectin was negative, and it probably indicates its lack of interfere in the gut differentiation. Also in the studies of Gotz, the reactions of this lectin have been similar to that of notochord and gut [4]. In their studies on human notochord, Odent, et al has claimed the reaction of LTA lectin with this organ to be positive, that is consistent with this study. These researchers have suggested the likelihood of presence of the mentioned terminal sugar in secreted Shh molecule [28]. Observation of positive reaction of notochord with WGA lectin also confirms the sialic acid expression to be severe in notochord, while it shows a general and mild view in other tissues. In the studies of Gotz, et al [4] as well as that of Loveless, et al [29] conducted using WGA lectin, the existence of sialic acid in notochord cells of human and bird embryos has been reported, which is consistent with results of this study. Since GSA1-B4 lectin, which identifies the terminal sugar of galactose, has not reacted with any of the studied organs, thus, the likelihood of the involvement of this sugar in the differentiation and interactions of these two organs is

negative. The glycoconjugates expressed in notochord may be related to the glycoproteins secreted by this organ, such as Shh, which acts as signaling molecule [30]. The other inductive molecules such as FGF, eFGF, and FGF-4 are also expressed in notochord [31]. Regarding the histochemical studies conducted on intestine, it should be noted that most studies have been conducted on after birth samples and fetal cases have been less discussed. Among the few cases which have discussed this issue, the studies of Jones, et al can be mentioned, which has studied endoderm layer covering the yolk sac of human embryos using 23 different lectins. In this study, different types of terminal sugars are identified in this layer and the researchers have attributed it to the severe metabolic and synthetic activities of this layer [32]. In the studies of Bryk, et al, which have been conducted on chicken embryos and newborn chickens, the severe reaction of endoderm layer to SBA lectin has been shown, which indicates the involvement of GalNac terminal sugar in gut differentiations and is consistent with the findings of this study [33]. In the studies of Gorge, et al, which have been conducted on newborn pigs, the expression of various glycoconjugates in the intestine of these animals is shown. The researchers have found that the incidence of sugar compounds in the intestinal enterocytes will be affected by intestinal microbial flora and in comparing to the stored samples; their expression severity will be increased at sterile conditions and has a positive correlation with the weight growth of these animals [34].

Since there are differences between appearance of various terminal sugars in notochord and the developing gut as well as their expression severity in this study with other studies, it should be noted that the difference of lectin histochemical reactions in different species of animal is a well-known issue [8].

Therefore, the glycosylation pattern in various species is different and this could justify the observed differences. Despite the use of limited number of lectins in this study, which is due to the expensiveness and the unavailability of specific inhibitors of each of the terminal sugars in the studied organs, the above results show that a variety of glycoconjugates with different terminal sugars are expressed in notochord

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and developing gut and given these findings, the following results will be concluded.

1- Notochord is a highly glycosylated organ in which different types of glycoconjugates with different terminal sugars such as; GalNac, Fucose and sialic acid are expressed. Whereas a variety of cellular processes such as proliferation, cellular rearrangement (conversion from epithelial mode into mesenchymal mode), differentiation and programmed death are observed in notochord, therefore, expression and appearance of various types of sugars in it, is natural. These findings are consistent with that of Gotz et al and Quondamatteo et al [4, 9].

2- Probably GalNac terminal sugar which reacts with the three specific lectins used both in notochord and primary gut, is involved in the differentiation of notochord and endoderm embryonic germinal layer to from primary gut epithelium, especially the differentiation of different types of its cells as well as notochord inductions on this organ. In the study of Gotz et al, the similarity of glycosylation pattern to embryonic notochord and primary gut and the possible involvement of these glycoconjugates in the interactions between these two organs have been also reported [4].

3- Any signaling molecule such as Shh, which is involved in the induction of endoderm by notochord, probably has a glycoprotein combination and terminal sugar of these compounds can be GalNac.

4- The terminal sugars expressed in notochord such as GalNac, Fucose and sialic acid are likely to be involved in differentiation of this organ, but the terminal sugars of galactose will not be involved in this process.

5- The terminal sugars of galactose, fructose and sialic acid will not be probably involved in endoderm induction by notochord.

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