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Original article

Lectin Histochemical Study of Vasculogenesis During Rat Pituitary Morphogenesis

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Abstract

Objective(s)
The aim of this study was to investigate glycoconjugates distribution patterns as well as their changes during the course of pituitary portal vasculogenesis and angiogenesis.

Materials and Methods
Formalin fixed paraffin sections of 10 to 20 days of Sprague Dawly rat fetuses were processed for histochemical studies using four different horseradish peroxidase (HRP) conjugated lectins. Orange peel fungus (OFA), Vicica villosa (VVA), Glycine max (SBA) and Wistaria floribunda (WFA) specific for α-L-Fucose, D-Gal, α, ß-D-GalNAc and D- GalNAc terminal sugars of glycoconjugates respectively.

Results
Our finding indicated that adenohypophysal cells reacted with OFA on gestational day 10 (E_{10}) and increased progressively to E_{14}. Staining intensity did not change from days 14 to17, then after increased following days to E_{20} significantly ($P< 0.05$). A few cells around Rathke's pouch reacted with VVA on E_{13}, increased to E_{14} and decreased significantly afterward ($P< 0.05$). Reaction of some cells around Rathke's pouch reacted with SBA on E_{14}. This visible reaction was the same as E_{18} and decreased later ($P< 0.05$). Many cells around Rathke's pouch reacted with WFA on E_{13} and increased on E_{14} and E_{15} and decreased thereafter ($P< 0.05$).

Conclusion
Reactions of OFA and other tested lectins with endothelial cells around Rathke's pouch and developing pars distalis were different. These results suggest that embryonic origin of hypophiseal pituitary portal (HPP) system endothelial cells are not the same and our finding also indicated that glycoconjugates with terminal sugars α-L-Fucose, D-Gal, α, ß-D-GalNAc may play critical role(s) in cell interactions and tissue differentiations such as vasculogensis and angiogenesis as well as other developmental precursors in formation of the pituitary gland.

Keywords: Angiogenesis, Glycoconjugates, Lectin, Pituitary, Rat

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Introduction

Vasculogenesis is the initial *de novo* stage of vasculature formation, and occurs in early developmental stages in which endothelial precursor cells or angioblasts derived from nascent mesodermal cells giving rise to the main blood vessels whereas the term angiogenesis denotes to the process of formation, branching and extension of new blood vessels from pre-existing capillaries already present in the tissues and organs (1, 2). These phenomena are necessary for development and embryogenesis as well as wound healing and reproduction in adult (1). Genetic analysis of vertebrates reveals that vascular endothelial growth factor (VEGF) plays essential role throughout embryonic vascular development (3, 4). Both angiogenesis and vasculogenesis are mediated by several phenomena such as inductions, cellular interactions and vascular microenvironments (5).

Glycosylation is an important post-translational modification of proteins involved in cell-cell interactions during embryonic development. Specific carbohydrate moieties of the oligosaccharide side chains of the glycoconjugates are among the factors involve in these interactions and developmental morphogenic processes are correlated with changes in the sugar content of glycoconjugates located on cell surfaces or in extracellular matrix (6). Moreover glycoconjugates that are present in the exteracellular matrix are involved in regulatory endothelial cell migration and angiogenesis directly and particularly play critical role(s) during early embryonic vasculogenesis (7).

It is classically admitted that the cells of connective tissue and endothelial cells of the pituitary capillary network originate from the mesodermal tissue (8). Moreover, in rats, the portal system could originate from the diencephalic vessels (angiogenesis) (9). In chickens, however another origin has been demonstrated for these cells: they are originated from mesencephalic neural crests and these cells differentiate to sinusoid endothelial cells in pars distalis (vasculogenesis) (10). In this regard, the origin of the endothelial cells of pituitary portal system is unclear and further studies are needed to clarify the origin of endothelial cells in different parts of pituitary portal system and roles of glycoconjugates during blood vessels formation.

Lectins are specific carbohydrate- binding proteins of non-immune origin that have proven utility for visualization of blood vessels and are valuable tools for the isolation of endothelial cells (11, 12). Some lectins react with terminal sugars of glycoconjugates on neural crest cells surfaces specifically (13). Other fucouse- binding lectins such as UEA-1, react with the vascular endothelial cells in all human tissues but the same lectins don't react with the vascular walls in many other animal species (14). Moreover, SBA lectin (sugar specifity N-acetylgalactosamine, β-D galactose) reacts with the developing endothelial cells during early vasculogenesis of the CNS in the 10-12 days old mouse embryos (15). In addition, endothelial cells bind to PNA and SBA lectins during cyclic ovarian angiogenesis in bovine (15, 16). However, it has been considered that the carbohydrate chains of glycocnjugates on developing endothelial cells surface are species – specific (16).

In the present study particular attention was paid to the origin and development of the pituitary portal system regarding to glycoconjugates distribution patterns and their changes during pituitary development by means of lectin histochemistry technique.

Materials and Methods

Thirty virgin adult female Sprague Dawly rats were used in this study. The animals were maintained at the animal house under controlled conditions (12 hr light and dark cycle, 21 °C and 50% relative humidity) with laboratory chow and water provided *ad libitum*. Then they were mated overnight with 15 fertile males of the same strain. The day on which spermatozoa were found in a vaginal smear was designated as embryonic day 0 (E_0).

At various gestational days from E_{10} to E_{20} pregnant rats were anesthetized and their fetuses were removed and sacrificed. The head of the fetuses were immediately washed in normal saline and fixed in normaline fixative consisting 10% formaldehyde in 0.01 M phosphate buffered saline (PBS) overnight at room temperature. After fixation, the tissue blocks were dehydrated with an ascending ethanol series, cleared with xylene and then embedded in paraffin with different orientations. The paraffin blocks were cut into sagittal and coronal serial sections of 5 µm thickness (17, 18).

The serial sections were deparaffinized with xylene, rehydrated through descending concentrations of ethanol and rinsed for 10 min in 0.1 M PBS. In order to block endogenous peroxidase, all the sections were placed in a methanol / H_2O_2 solution (1:100) for 45 min in dark and then were treated with PBS solution for 30 min at room temperature (19-21). Four horse radish peroxidase (HRP) labeled lectins, which were purchased from Sigma Aldrich Company, were diluted to reach final concentration 10 µg/ml of lectin in 0.1 M PBS. Thereafter, five sections were chosen randomly and incubated with each lectin in a humid chamber for 2 hr at room temperature. The tested lectins and their sugars specificity has been listed in Table 1. After incubation, the sections were washed extensively with PBS for 3 min and treated with DAB solution (0.03 g DAB in 100 ml PBS and 200 µl H_2O_2/100 ml PBS) for 15 min at room temperature in dark (19-21). After being washed in running water, all the sections were counterstained with a 1% solution of alcian blue at pH 2.5 for 1 min. Finally the sections were dehydrated in increasing graded ethanol, cleared in xylene and mounted in glass slide. In order to detect staining intensity, the reactions of the tested lectins were observed by three examiners separately with Olympus AH-2 microscope (21). On the basis of staining intensity, sections were graded and Kruskal-Wallis non-parametric statistical test was used to compare differences between samples.

This experimental research was done in 2007 in Mashhad University of Medical Sciences according to ethics committee guidelines and all protocols of animal experiments have been approved by the Institution's Animal Care Committee.

Results

The development of the pituitary gland and its blood vessels in rat embryos occur from gestational day 10 (E_{10}) through day 20 (E_{20}). In this study our finding showed that the reaction of some developing adenohypophysal cells with OFA started from gestational day 10 (E_{10}). This reaction which was visible in basement membrane as well as in the Golgi zone of epithelial cells of Rathke's pouch (Figure 1), increased on E_{14}. From days E_{14} to E_{17}, the staining intensity was the same and then increased during differentiation on the following days (Figure 2) ($P< 0.05$). In contrast, binding site for OFA lectin could not be observed around the developing Rathke's pouch mesenchyme during pituitary portal system development (Table 2).

As developmental stages proceed, anterior wall of the Rathke's pouch becomes thickened and more elongated, enveloping areas of vascularized mesenchyme (Atwell region), to form pars distalis. A few cells of developing pars distalis and the dorso-ventrally region around the Rathke's pouch (Atwell region) reacted with VVA from E_{13} and increased to E_{14} (Figure 3) and decreased afterward significantly ($P< 0.05$). Although reactions of developing pars distalis and the dorso-ventrally region around the Rathke's pouch (Atwell region) showed the same pattern, regulated intensity reactions of VVA with developing pars distalis was weaker compared to intensity reactions of atwell's region (Tables 2, 3).

Some cells around Rathke's pouch reacted with SBA from E_{14} (Figure 4) and to E_{18} were the same and then decreased afterward ($P< 0.05$). SBA reaction with the developing pars distalis was not observed (Figure 4 and Tables 2, 3). A few proliferating and differentiating cells of anterior wall of Rathke's pouch as well as many mesenchymal cells around Rathke's pouch, especially Atwell's recess, reacted with WFA from E_{13} and increased on days E_{14} (Figures 5, 6) and E_{15}

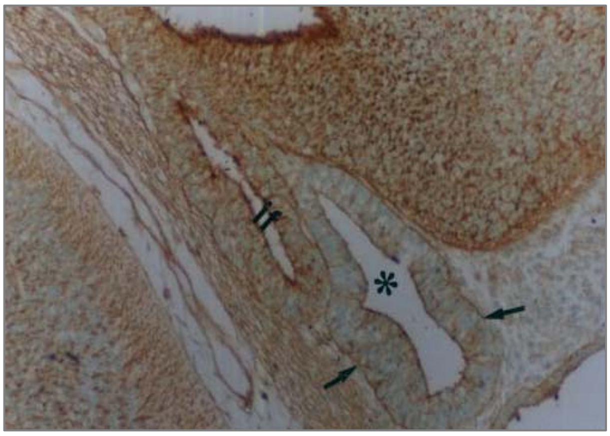

Figure 1. Sagital section of developing pituitary gland on day 10 of gestation, incubated with OFA. Rathke's pouch basement membrane (arrow) shows reaction moderately. Golgi zone reaction is weak. Rathke's pouch (star), stomodeum cavity (O), infundibulum (if), magnification= ×220.

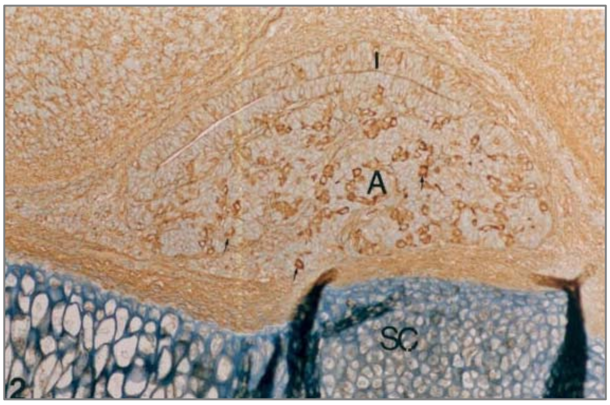

Figure 2. Sagital section of developing pituitary gland on day 18 of gestation, incubated with OFA. Some cells reacted with OFA strongly (small arrow): these cells are differentiated into the endothelial cells. Anterior wall of Rathk's pouch or developing pars distalis (A), intermediate lobe (I), magnification= × 400.

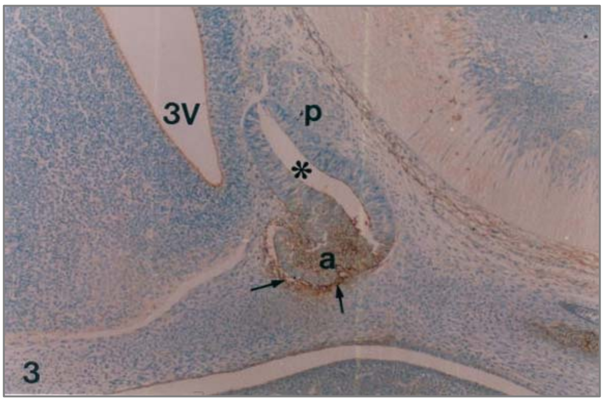

Figure 3. Sagital section of developing pituitary gland on day 14 of gestation, incubated with VVA. A few cells around Rathke's pouch reacted with VVA (arrow) strongly. Rathke's pouch cavity (star), stomodeum cavity (O), Anterior wall of Rathk's pouch or developing pars distalis (A), intermediate lobe (I), posterior lobe (p), 3rd ventricle (3V), magnification= × 100

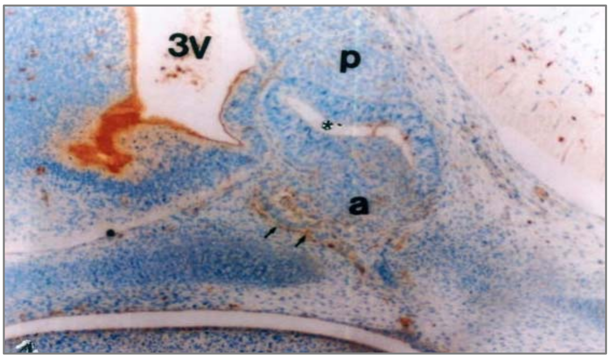

Figure 4. Sagital section of developing pituitary gland on day 14 of gestation, incubated with SBA. Some cells around Rathke's pouch reacted with SBA (arrow) moderately. Rathke's pouch cavity (star), stomodeum cavity (O), anterior wall of Rathk's pouch or developing pars distalis (A), intermediate lobe (I), posterior lobe (p), 3rd ventricle (3V), magnification= × 100

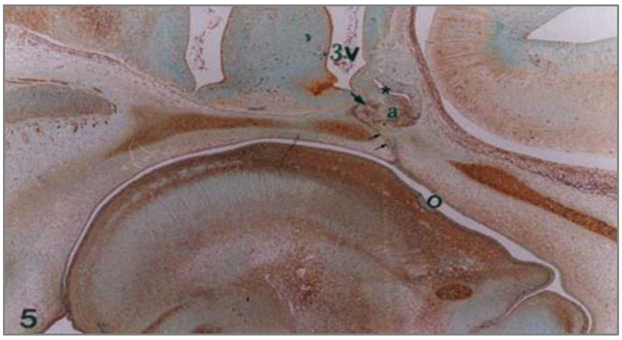

Figure 5. Sagital section of developing pituitary gland on day 14 of gestation, incubated with WFA. Many mesenchymal cells around Rathke's pouch, sub epithelial tissues of the stomodeum roof and craniopharyngeal duct reacted with WFA (small arrow) strongly. These reactions also were visible in Atwell's recess (arrow). Rathke's pouch cavity (star), stomodeum cavity (O), anterior wall of Rathk's pouch or developing pars distalis (a), intermediate lobe (I), 3rd ventricle (3V), magnification= × 40

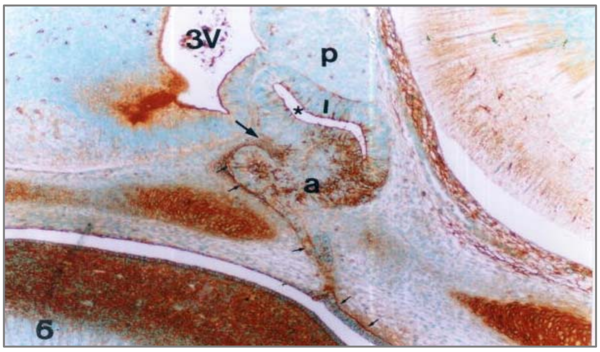

Figure 6. Sagital section of developing pituitary gland on day 14 of gestation, incubated with WFA. Many mesenchymal cells around Rathke's pouch, sub epithelial tissues of the stomodeum roof and craniopharyngeal duct reacted with WFA (small arrow) strongly. These reactions also were visible in Atwell's recess (arrow). Rathke's pouch cavity (star), stomodeum cavity (O), anterior wall of Rathk's pouch or developing pars distalis (A), intermediate lobe (I), posterior lobe (p), 3rd ventricle (3V), magnification= × 100.

Table 1. List of lectins used in the study and their main sugar specificities (17).

Lectin	Abbreviation	Carbohydrate – binding specificity
Aleuria aurantia(Orange fungus)	OFA	α- L –Fucose
Glycin max (Soybean)	SBA	α ,ß-D-GalNAc>D- Gal
Vicia villosa (Hairy winter vetch)	VVA	GalNAc
Wistaria floribunda	WFA	D- GalNAc

Table 2. The median of staining intensity of Atwell region mesenchyme around Rathke's pouch, the developing vascular region, reacted with different lectins.

Tested lectins				Gestational days
WFA	SBA	VVA	OFA	
-	-	-	-	10-12
+	-	+	-	13
++	+	+++	-	14
+++	+	+	-	15
++	+	-	-	16
+	+	-	-	17
-	+	-	-	18
-	-	-	-	19
-	-	-	-	20

Table 3. The median of staining intensity of developing anterior pituitary, anterior wall of Rathke's pouch, reacted with different lectins.

Tested lectins				
WFA	SBA	VVA	OFA	Gestational days
-	-	-	+	10-12
+ -	-	+	+	13
+	-	++	++	14
++	-	+	++	15
++	-	-	++	16
+	-	-	++	17
-	-	-	+++	18
-	-	-	+++	19
-	-	-	+++	20

(P< 0.05). These reactions were also visible in the subepithelial tissues of the stomodeum roof and craniopharyngeal duct. Above pointed reactions decreased afterward (P< 0.05) and finally disappeared on days E_{18} and E_{20}.

Discussion

The pituitary gland originates from two different embryonic origins. Anterior and intermediate lobes are derived from the oral ectoderm via formation of Rathke's pouch, located to the oropharyngeal membrane rostrally, which is seen in the 24 day-old human embryo and in the 10-11 day-old rat embryo and the posterior lobe from the neural ectoderm of developing diencephalons (22).

The development of angiogenic features is a complex process and involves the ability of the endothelial cell to break homotypic cell contacts, migrate through basement membrane and extracellular matrix, proliferate and reorganize to give an intact neovessel (23).

The pituitary gland vascularization develops from the surface network covering prosencehpalic vesicle (angiogenesis). A fine meshwork of vascular plexus surrounds the evaginating processus infundibularis at E_{12} and Rathke's pouch, which is visible at this time, acquires its blood from the above-mentioned plexus (24). The vascularization of the stomodeal roof around Rathke's pouch starts on E_{13}, also providing vessels for Rathke's pouch (25). At this time (E_{13}) a developing meshwork of vessels is visible at the anterior surface of Rathke's pouch. In our study these regions reacted with VVA and WFA, which are specific for GalNAc glycoconjugates terminal sugars with different linkage to their penultimate sugars. The primary portal veins, the vascular network of the floor of the diencephalic vesicle with that of the pars distalis, were clearly seen at E_{14}. Since development of the stomodeum roof vessels starts on E_{13}, their connection to Rathke's pouch could be first observed clearly on E_{14}. In our study these regions reacted with WFA and SBA lectins. SBA lectin is specific for β, α -D-GalNAc. Primary portal veins pass through Atwell's recess into the sinusoids of pars distalis. In our study, Atwell's recess reacted with VVA and WFA strongly on E_{14}. According to these data, we suggest glycoconjugates with terminal sugar D-GalNAc react with different terminal linkage and special position, which are detected by SBA, WFA and VVA lectins, and may play critical role(s) during pituitary portal system angiogenesis. The endothelium is the first component of the blood vessels to develop and determines the pattern of the vasculature (26-28). Two of the major challenges in the study of vascular development are to resolve the origin and mode of determination of the endothelium, and to establish the mechanism of patterning and morphogenesis that lead to the formation of blood vessels in their appropriate positions (29). Sinusoid plexus in pars distalis appears at E_{15}, and vascular density increases significantly on E_{16}, while the branches of the vascular plexus covering the ventral surface of the diencephalons become slightly or moderately thicker than elsewhere on the surface of the brain. After days E_{17}-E_{21}, there are no major changes in the distribution of blood vessels of the pituitary–median eminence complex (9). The cells were differentiated to the endothelial sinusoidal cells in pars distalis, which reacted with OFA lectin with specific spatiotemporal pattern. In addition, α-L–Fucose- binding OFA reacted with neural crest cells (13). Therefore, in the chicken, it has been suggested that angioblasts cells originate from mesencephalic neural crests and these cells differentiate to sinusoid endothelial cells in pars distalis (9), a process which is named vasculogenesis. Angiogenesis involves the most dynamic functions of the endothelium. In response to an angiogenic stimulus, endothelial cells in principal vessel separate from each other, leaving uncovered segments of the basement membrane and migrate while other endothelial cells proliferate (29). Migrating and proliferating cells form loops and then tubes of the basement membrane communicates with that of the principal vessel. This formation of sprouts continues until the necessary microvascular network is formed (30).

Our results show that sinusoid endothelial cells may be originated from neural crest cells and we are able to confirm suggestions offered regarding this matter.

Therefore, OFA binding structures are oligosaccharides, which may be important for cellular interactions during morphogenetic processes. In this regard, regulated appearance and glycoconjugates changes such as α-L-Fucose are required for PPS vasculogenesis. Therefore, glycoconjugates with terminal sugars α-L–Fucose and α, ß-D-GalNAc are also expressed in maturing microvessels during pituitary development. This finding correlates well with the idea of early vasculogenesis and continued vascular plasticity and lead to PPS formation.

Conclusion

The results suggest that mentioned glycoconjugates are expressed around the Rathke's pouch, areas of vascularized mesenchyme, in which form hypophyseal microvessels. However, the role(s) of glycoconjugates is unclear in angiogenesis but it seems that the glycoconjugates on the cell surface and extra cellular matrix might be involved in regulation of embryonic pituitary gland development as well as its angiogenesis and vaculogenesis. Further studies are needed to demonstrate precise role of glycoconjugates during pituitary development, origin of portal system and comparison of angiogenesis and vasculogenesis by using lectin histochemical and immunohistochemical techniques.

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