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The localization and expression of HNK-1-immunoreactive molecules in chicken embryos

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ABSTRACT. HNK-1 (Leu-7 antigen) is a unique carbohydrate moiety bound to the subsets of a number of cells and extracellular matrix glycolipids and glycoproteins, acting as an adhesive. Using immunohistochemistry and Western immunoblot method, we observed the localization and expression of the HNK-1 epitope in chicken embryos at different developmental stages. The results of immunohistochemistry show that HNK-1-immunoreactivity (HNK-1-IR) is distributed extensively in different tissues, and the intensity of immunoreaction varies with the development stages in chicken embryos. In the nervous system, the HNK-1-IR decreases gradually in brain but remains almost stable in spinal cord with embryo development, and it is strong in the neural tube and the neural crest at stage 11. At later stages, all forming ganglia are labeled. The special conductive tissues of the heart are also recognized by the HNK-1 antibody. In the alimentary tube, HNK-1-IR mainly appears in the Auerbach plexus, the Meissner plexus, and the mucosal membrane. Endothelial cells, pit cells and/or fat-storing cells may contribute to the HNK-1-IR of the liver. The results of Western blot show that there are at least three kinds of HNK-1-reactive molecules expressed in different tissues of different stages, and the kinds of these molecules remain relatively identical with developmental stages. Our results from chicken embryos demonstrate that HNK-1 epitope is expressed widely in the entire embryonic period, and the distribution of HNK-1-positive molecules shows histological specificity.

KEY WORDS : HNK-1-immunoreactivity, development, epitope, chicken embryo, immunohistochemistry, Western immunoblot.

INTRODUCTION

The monoclonal IgM (immunoglobulin M) antibody HNK-1, which recognizes a carbohydrate epitope, was first generated to Human Natural Killer cells (Abo & Balch, 1981). In 1983, Schuller-Petrovic, et al. found that the nervous system shared HNK-1 antigenic determinants with lymphocytes. Thereafter, HNK-1 antibody became a valuable tool in studying the developing nervous system and other systems (Holley & Yu, 1987 ; Nordlander, 1989 ; Metcalfe et al., 1990).

The HNK-1 epitope is a 3'-sulfated gulcuronyl-substituted-lactoseries oligosaccharide bound to certain glycolipids (Ilyas et al., 1984), proteoglycans (Margelis et al., 1987) and subsets of a number of cells and extracellular matrix adhesion molecules. The glycolipid (Chou et al., 1986) and glycoprotein (Voshol et al., 1996) both have the sulfate-3-GlcAβ1→3Gal1→4GlcNAc domain at the non-reducing end. There are a number of cell adhesion molecules in the nervous system representing the HNK-1-immunoreactivity (HNK-1-IR), including neural cell adhesion molecules (N-CAMs), L1 (Kielhauer et al., 1985), myelin-associated glycoprotein (MAG) (McGarry et al., 1983), P0 (Bollensen & Schachner, 1987), peripheral myelin protein 22 (PMP22) (Snipes et al., 1993), J1 (Kruse et al., 1985), ependymins (Lakos et al., 1994), sulfoglucuronyl glycolipids (SGGL) (Chou et al., 1987), etc. The multiple expression of the HNK-1 epitope is involved in cell-cell or cell-extracellular matrix recognition during various choreographed stages of cell proliferation, migration, differentiation and maturation. Moreover, these molecules play an important role in learning and memory (Schmidt et al., 1995 ; Pradel et al., 1999). Most of the HNK-1-reactive molecules are expressed only in embryos or in very early postnatal stages, and restricted to a particular stage in adults.

The HNK-1-IR is distributed on the surface of migrating neural crest cells and their derivatives during development (Vincent et al., 1983 ; Bronner-Fraser et al., 1986 ; Sadashiani et al., 1990)). This indicates that the HNK-1 epitope engages in the organization of nervous system directly or indirectly by the cell adhesion molecules in vertebrates. For HNK-1, there are many reports about the migratory routes of the neural crest cells and the factors that influence migration in the embryos (Bronner-Fraser, 1986 ; Hirata et al., 1997). Although some authors also studied embryos, they devoted their attention to the cell level or some specific tissue (Metcalfe et al., 1990 ; Sakai, et al., 1994 ; Blom, et al., 1999). Many molecules have HNK-1-IR during development, but little is known about the expression of HNK-1 epitope throughout the entire embryonic development. Although the location of the HNK-1 can be ascertained by immunohistochemistry, this method cannot distinguish which kind of HNK-1-positive molecule is expressed. Moreover, it is not clear whether only one class or different classes of these molecules function at a certain stage. Here we studied HNK-1 immunoreaction using immunohistochemistry combined with Western blot technology in the different stages of chicken embryos. These results provide some evidence for understanding the roles of HNK-1 during embryonic development.

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MATERIAL AND METHODS

Animals and tissue preparation

Fertile White Leghorn chicken eggs were obtained from commercial sources and incubated in an auto-incubator (Grumbach, made in Germany) at 37.5˚C-37.8˚C and 60% relative humidity. The embryos were used after different incubation times, divided into five experimental groups : 39 hours, 3-4, 7.5, 11.5 and 15 days. According to the morphological stage series of HAMBURGER & HAMILTON (1951), the above groups were named as stages 11, 20, 32, 37-38 and 41, respectively. Three to five embryos were used in each group.

The embryos were fixed for 4-24hr in cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The preparations were then washed, dehydrated in ascending graded alcohols, cleared in xylene, and embedded in paraffin. Transverse or longitudinal 8-10μm paraffin sections were made and every sixth section collected on a slide with gelatin-chrome alum. Each slide was deparaffinized through xylene and descending graded alcohols, and then rinsed with distilled water.

Immunohistochemical staining

After pretreatment with hydrogen peroxide (H_2O_2), the above slides were incubated in normal goat blocking serum and then in a 1 :10 dilution of mouse HNK-1 IgM antibody overnight at 4˚C. Following thorough rinsing, biotin-labeled goat anti-mouse IgM (Sigma, 1 :200) was applied, followed by ABC (avidin-biotin complex ; Vector products) solution at room temperature. Finally, the sections were reacted with a mixture of diaminobenzidine (DAB, Sigma) and H_2O_2 until the reaction product was clearly visible. Sections were rinsed in water, dehydrated in ascending ethanols, cleared in xylene, mounted in Canada balsam and coverslipped. Some sections were lightly counterstained with hematoxylin and eosin.

For control, the primary antibody was omitted, and replaced by normal goat serum.

Western immunoblot

Based on our immunohistochemistry data in this experiment, we chose three stages (20, 32 and 37-38) for Western blot. Fourteen embryos were used from each stage. The sample protein was extracted from the whole embryos in stage 20, and from the brain and the trunk of embryos in the other stages, with 5 volumes of 0.05mol/L Tris-HCl buffer, pH7.5, containing 10mM EDTA, 1% sucrose, 2% Triton X-100, 100mM NaCl and 1mM PMSF. Homogenates were centrifuged for 1hr at 10,000g at 4˚C, and supernates collected and stored at -70˚C. Protein determinations were carried out according to LOWRY et al. (1951) using bovine serum albumin (BSA) as standard. Protein samples were heated for 5min at 100˚C in sample buffer containing mercaptoethanol and sodium dodecyl sulfate, and electrophoresed (BIO-RAD) on 7.5% resolving gel in the discontinuous buffer system of LAEMMLI (1970). Approximately 100 μg of total protein was loaded in each lane. For immunoblot, proteins were transferred onto nitrocellulose membrane in 25mmol/L Tris, 192mmol/L glycine and 20% methanol, pH8.3. After non-specific protein binding was blocked with 10% goat serum, the nitrocellulose membrane was incubated successively with HNK-1 antibody (1 :20), biotin-goat anti-mouse IgM, and ABC kit. Finally, colour development was achieved with H_2O_2 and DAB. The marker bands were obtained from the same gel and stained with Coomassie Brilliant Blue R-250.

RESULTS

The localization of HNK-1-immunoreaction

At stage 11, HNK-1-positive neural crest cells were detected at the medial and dorso-lateral sides of somites, from where these cells initiated their migration to the ventral trunk. Moreover, HNK-1-immunoreactive cells were ubiquitously distributed along the rostro-caudal axis at the neural tube level (Fig. 1A), and the HNK-1-reactive fibres were distributed on the wall of the neural tube (Fig. 1B).

At stage 20, labelled cells or materials were scattered in the migratory pathway of the crest cells (Fig. 1C). All ganglia derived from the neural crest, including the ganglia of cranial nerves, spinal ganglia and sympathetic ganglia, were labeled, and were as compact as crest cells (Fig. 1B). Also, HNK-1-IR was found in the conductive tissue of the heart (Fig. 1D) and dotted around the notochord (Fig. 1C).

At stage32, the HNK-1-IR was the same as that in stage 20 in the conductive tissues of the heart and in the ganglia.

Compared with the brain, the intensity of HNK-1-IR in the spinal cord was relatively stable in different stages. The spinal cord has three morphological parts : the substantia alba, the substantia grisea and the central canal (Fig. 2A). The HNK-1-IR was stronger in the substantia alba than in the substantia grisea (Fig. 2B). At stage 20 HNK-1-IR was around the notochord, but concentrated on a narrow area of the ventral notochord at stage 32 (Fig. 2B). The antibody also stained fibres innervating limbs (Fig. 2C) and the vicinity of blood vessels (Fig. 2F). In the alimentary tube, the HNK-1-IR was distributed in the Auerbach plexus (myenteric plexus), the Meissner plexus (plexus submucosus) (Fig. 2D) and the mucus membrane, and was also scattered in the liver (Fig. 2E).

At stage 37-38, the intensity of HNK-1-IR in the brain was weaker than at early stages, but in the trunk it was similar to that at stage 32 (Fig. 2F). No HNK-1-IR was observed in the brain at stage 41.

We also investigated localization of the HNK-1 epitope in the brains of chickens at postnatal days 1 and 3, and of adult white-rumped munia (*Lonchura striata swingoei*). No reactive cells or fibres were observed in these cerebrums.

In controls, where only normal goat serum was used, no specific staining was observed.

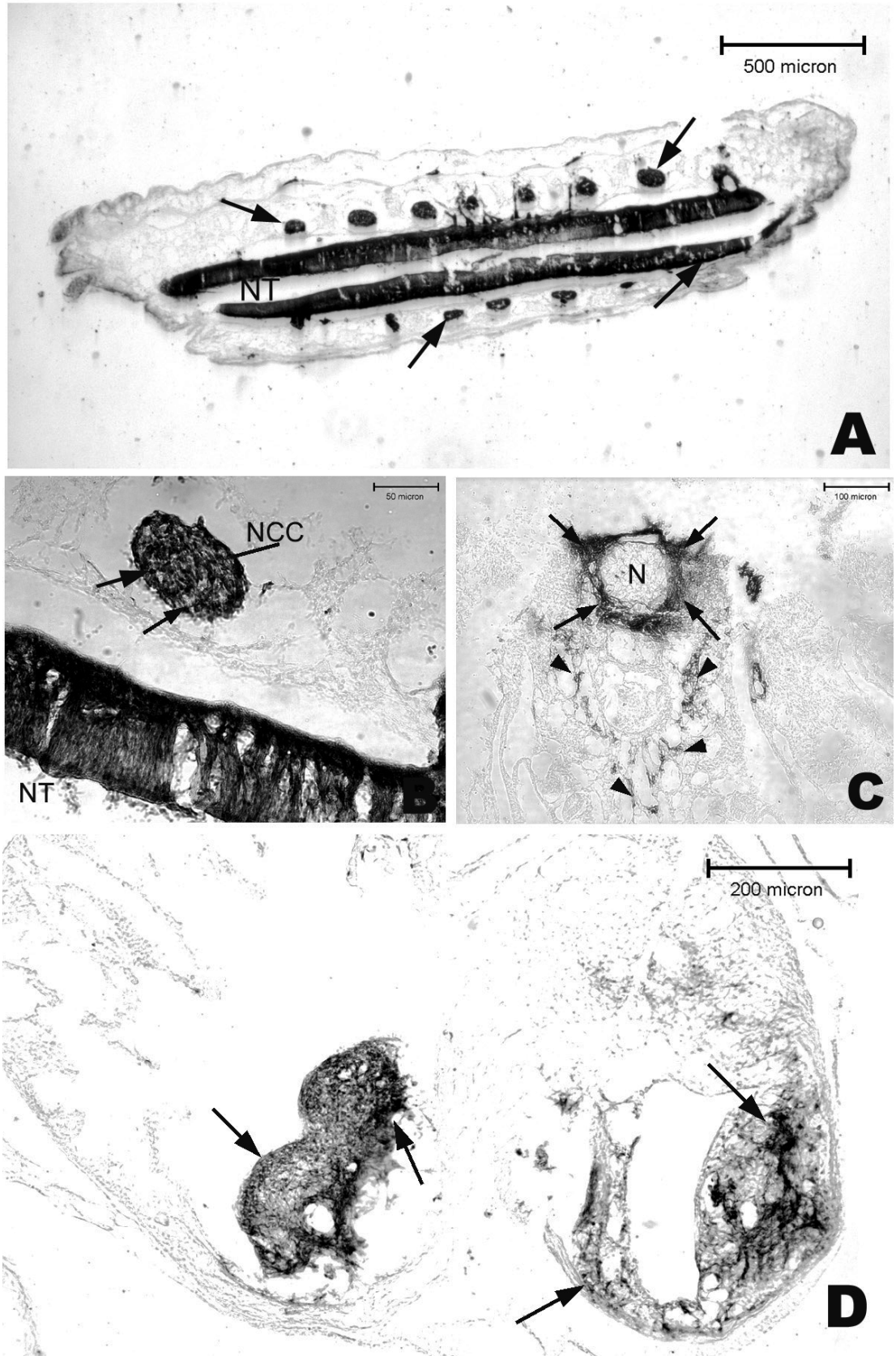

Fig. 1. – A. Whole mount of the chicken embryo at stage11. The arrows indicate where HNK-1 antibody labels the neural tube and neural crest. – B. High-power micrograph of a whole mount of a chicken embryo at stage 11. Arrows show two HNK-1-positive cells out of many. – C. Transverse section through the notochord at stage20. Large arrows indicate the surrounding of the notochord and small arrows indicate the ventral migratory pathway of the neural crest cells. – D. Longitudinal section of the heart at stage20. Arrows show HNK-1-positive conduction system of the heart. (NT: neural tube; NCC: neural crest cell; N: notochord).

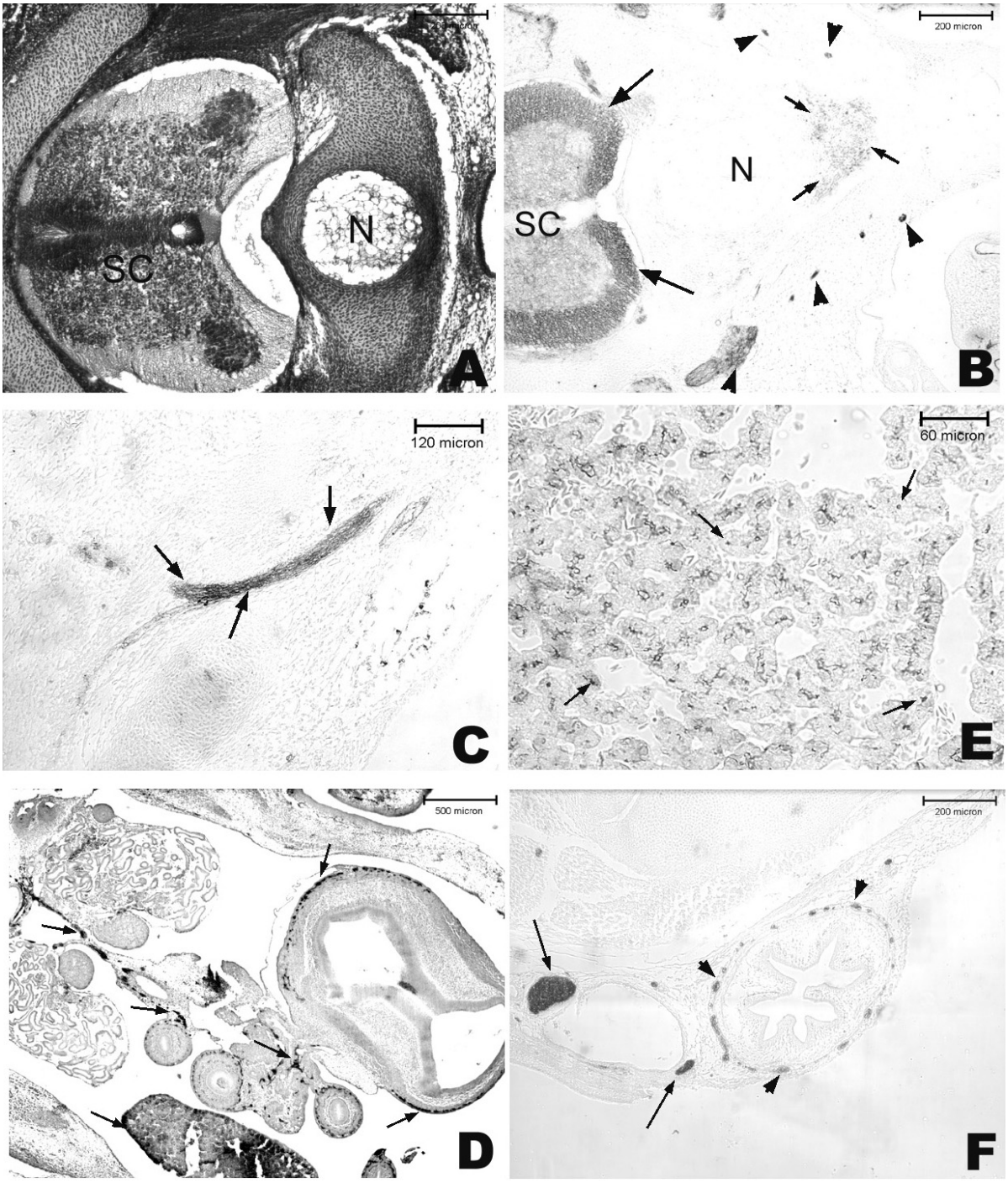

Fig. 2. – A. Transverse section of chicken embryo through spinal cord at stage32, stained with hematoxylin and eosin . It shows three parts of the neural tube: the substantia alba, the substantia grisea and the central canal. – B. Transverse section through the spinal cord at stage32. HNK-1 antibodies label the spinal cord (large arrows), the ventral side of the notochord (middle arrows) and the ventral migratory pathway of the neural crest cells (short arrows) . – C. Transverse section of the trunk of chicken embryo at stage32. HNK-1 antibodies label the nerve of the limb (arrows). – D. Transverse section of the trunk of chicken embryo at stage32 (hematoxylin-counterstained). Arrows show HNK-1-positive local nervous system in the mucosa of the alimentary tube. – E. Section through the liver of chicken embryo at stage32 (hematoxylin-counterstained). Arrows show some of the many HNK-1-positive products. – F. Transverse section of the trunk of chicken embryo at stage37-38. HNK-1 antibody labels the gut (short arrows) and the vicinity of the blood vessel (long arrows). (N: notochord; SC: spinal cord)

Western immunoblots

The results showed that there were more than three kinds of immunoreactive molecules at all the stages we investigated (Fig. 3). Moreover, from stage 20 to stage 37-38, the size of molecules bearing the HNK-1 epitope remained the same. The immunoblot showed the appearance of labeled protein bands at 40, 80 and approximately 110-200kDa. The most prominent band had a molecule size of approximately 80kDa. Close to this band, there was anoth-

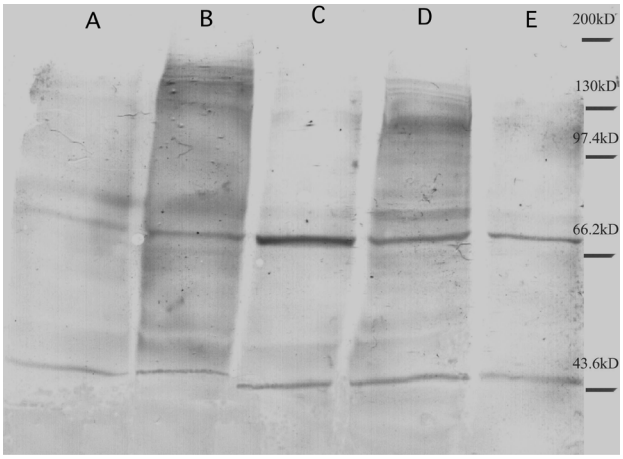

Fig. 3. – Western blot analysis of total proteins of the embryos
Lane A is blot obtained from whole embryos of stage 20; Lanes B and C are blots obtained from stage32 (B, from brain tissues; C, from trunk tissues); Lanes D and E are blots obtained from stage37-38 (D, from brain tissues; E, from trunk tissues); The five marker bands on the right were obtained from the same gel and stained with Coomassie Brilliant Blue R-250, corresponding to 200kD, 130kD, 97.4kD, 66.2kD and 43.6kD respectively from top to bottom.

er band stained very lightly in lanes A, B and D. In lanes C and E, the sites of this band were hard to see.

DISCUSSION

The spatial and temporal specificity of the HNK-1 epitope expression

Our results showed that HNK-1-IR in the brain decreased gradually with embryo development, and disappeared in postnatal and adult brains. This implies that the molecules with the HNK-1 epitope may play an important role in early development of the chicken brain. A similar tendency of HNK-1-IR was found in the 3-4 weeks postnatal mouse cerebrum (Schwarting et al., 1987). However, in the adult rat and mouse cortex, HNK-1-IR was expressed selectively on GABA-engic (GABA : γ-aminobutyric acid) neurons containing the calcium-binding parvalbumin (Kosaka et al., 1990 ; Ren et al., 1994). These observations indicate that the HNK-1 epitope engages in the organization of central nervous system, with species differences between rodents and birds during brain development.

During the development of the neural tube in chicken embryos, we found that HNK-1-IR was stronger in the substantia alba than in the substantia grisea at stages 11, 20, and 32, and stronger in the ventral substantia alba than in the dorsal side at stage 32. The substantia alba mainly consisted of myelinated fibres, and the substantia grisea consisted of non-myelinated fibres. This indicate that the myelin-forming Schwann cells mainly secrete HNK-1 adhesive molecules such as MAG, P0 and PMP22 etc. Martin et al. (1988, 1992) reported that HNK-1-positive molecules were preferentially expressed on motor neurons and motor nervous fibres in the ventral root of the spinal cord in mice, especially on the Schwann cells. By contrast, HNK-1-IR was represented both in ventral and dorsal roots in the lamprey (Hirata et al., 1997). In addition, we found that HNK-1-IR occurred in the limb nerves at later development. These investigations indicate that the HNK-1 epitope participates in the formation of myelin and the differentiation of motor neurons in spinal cord.

In conjunction with previous investigations (Bronner-Fraser, 1986 ; Loring & Erickson, 1987 ; Kuratani, 1991), our results revealed that migration of trunk neural crest cells in chicken embryos is also along ventral pathways. The same migration pattern was also identified in zebrafish (*Brachydanio rerio*) embryos (Raible et al., 1992) and in swordtail (*Xiphophorus helleri*) embryos (Hirata et al., 1997). In chicken and in *Xenopus* species (Sadaghiani & Vielkind, 1990), neural crest cells are present in the dorsal and lateral sides of the neural tube. They also migrate further to the ventral side of the notochord, and even to the area surrounding the dorsal aorta, where the sympathetic nervous system is formed. In the lamprey (*Lampetra reissneri*) on the other hand, Hirata et al. (1997) found no HNK-1-positive cells that migrate ventrally beyond the notochord at axial levels. These findings demonstrate that the migratory pathway of neural crest cells is relatively conservative in fish, birds and mammals.

In the heart of chicken embryos, Luider et al. (1993) and our results showed that HNK-1-IR is present in the septum of atriorum and ventriculorum where the special conductive tissues of the heart are located. The conductive tissues of the heart are not derived from neural crest cells, but these tissues displayed the HNK-1-IR in embryos. This phenomenon was also observed in rats (Nakagawa et al., 1993 ; Sakai et al., 1994 ; Wenink et al., 2000). Chuck et al. (1997) found that HNK-1-IR was more intense in His-Punkinje system than in normal myocardium in avian embryos. This shows that HNK-1-positive proteins implicated in cell interactions play an important role in the formation and maintenance of function of the cardiac conduction system. In human embryos with abnormal atrial automaticity, HNK-1-IR was found to be expressed temporarily around the left and right atrial pacemaker (Blom et al., 1999). This indicates that HNK-1 plays an important role in the development of conductive tissues of the heart.

The enteric nervous system, including the Auerbach plexus and the Meissner plexus etc., is derived from neural crest cells. As well as in the enteric nervous system of the alimentary tube, we also found HNK-1-IR in the mucus membrane and liver of chicken embryos. A similar phenomenon was observed in the vicinity of the hepatic sinusoid in rats (Peinad, 2000). The question is, which functions of these HNK-1-positive cells were active in the alimentary tube during development. It is known that many kinds of APUD (Amine Precursor Uptaking and Decarboxylating) cells are scattered and mingled with the endothelial cells of the alimentary tube and in the digestive glands, regulating digestion and metabolism. Margolish et al. (1987) found that some APUD cells were chromaffin cells, which expressed some HNK-1-positive glycoproteins and glycolipids. The HNK-1-positive cells in the alimentary tube of chicken embryos may, therefore, be APUD cells. In liver tissues there are four kinds of cells : endothelial, Kupffer, fat-storing and pit. Some endothelial cells can secrete an active substance with the HNK-1 epitope, such as chondroitin sulfate, and the fat-storing

cells are descendants of the neural crest cell. The pit cell is a large granular lymphocyte, and it possesses the activity of natural killer cells. It is possible that the HNK-1-IR in the liver of chicken embryos may be attributed to some secretory endothelial cells, fat-storing cells and/or pit cells.

The above indicates that HNK-1 can engage in organization of the nervous system, including the central and peripheral systems. It can influence the development of the conductive tissues of the heart, and the development of the system to regulate digestion and metabolism.

The multiple expressions of the HNK-1 epitope

Our results of Western immunoblot show that there are at least three kinds of HNK-1-reactive molecules participating in development of chicken embryos at each stage. The blots show a slight difference of HNK-1-positive molecular expression between brain tissues and trunk tissues. For example, 110-200kD bands could be detected clearly from the brain tissues, but were only faintly visible from the trunk tissues. Many more kinds of HNK-1-positive molecules are expressed in the brain than in the trunk at the same stage. It is very interesting to find that the same epitope was expressed on many kinds of molecules. This result is similar to that from avian dorsal root ganglia (DRG) with HNK-1 antibody (Tucker et al., 1984). In 11-day-old avian embryos, three major bands of 130, 200 and 300kD were detected from DRG, while a major diffuse band spreading from 300 to 200kD and two other components at 180 and 130kD were observed in the cerebrum. Moreover, some other antibodies, such as the monoclonal antibodies VC1.1 (Naegele & Barnstable, 1991), L3 (Kücherer et al., 1987), zn-12 (Metcalfe, et al., 1990) could similarly react with several molecules. These antibodies probably recognized one lapping, or identical carbohydrate epitopes. Generally, the molecules detected by these antibodies had weights ranging from 20 to 300kD, and our result tallies with this point. However, we do not yet understand the mechanism and the significance of overlapping expression from the different HNK-1 molecules, nor how these molecules interact with each other. It is likely that these molecules can be expressed at a precise time and in specific cells during the development of the nervous system. Future studies will need to determine and separate the different kinds of HNK-1-reactive molecules with specific antibodies, to determine which kinds of HNK-1-reactive molecule play different roles at different stages.

Our results revealed that the HNK-1 epitope is distributed quite widely in embryogenesis. Interestingly, we found that HNK-1-positive cells are accumulated not only in the neural crest at stage 11, but also in the ganglia at later stages. Thus, we infer that the molecules with HNK-1-IR could advance cell-cell adhesion or cell-matrix adhesion so as to contribute to cell accumulation and ganglion formation. These findings help us to understand the roles of HNK-1-positive molecules during development in the chicken embryo.

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