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Original article

L5: A carbohydrate epitope involved in neural development

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Summary – The L5 carbohydrate epitope is developmentally regulated in the vertebrate nervous system, both at very early stages (neural induction) and during postnatal development. Here, we review the evidence indicating that, during gastrulation, L5 may be a marker for cells competent to respond to neural inducing signals emanating from Hensen's node (the 'organizer') and that it may itself be involved in the response to neural induction. In postnatal cerebellar development, when L5 is found on astrocytes, it participates in the outgrowth of astrocytic processes on extracellular matrix components. Finally, we indicate the structural relatedness of the L5 carbohydrate and Le^x, CD15 and SSEA-1.

astrocyte / carbohydrate / cerebellum / CD15 / chick embryo / Hensen's node / $Lewis^x$ / mouse / neural induction / SSEA-1

Introduction

The L5 monoclonal antibody is one of a number of such antibodies that resulted from immunization of rats with L2/HNK-1 positive glycoproteins from mouse brain ('rest L2'; [18]), which was undertaken in an attempt to identify new members of the L2/HNK-1 family of cell adhesion molecules and to investigate their functional properties in early neural development. It was shown that the L5 epitope is expressed by the cell recognition molecule L1 (Ng-CAM) and Thy-1 and most prominently by the chondroitin sulphate proteoglycan astrochondrin, present on astrocytes [38].

Since several different glycoproteins were immunoreactive with this antibody, it appeared that the antibody might react with a carbohydrate epitope on these molecules. Indeed, a recent detailed analysis [40] established that the L5 antibody recognizes a carbohydrate structure closely related to the 3-fucosyl-N-acetyllactosamine sequence, which is known as Le^x, CD15, X-hapten or stage-specific embryonic antigen 1 (SSEA-1).

The expression of the L5 epitope is developmentally regulated in the nervous system, both at very early stages (neural induction) [31, 39] and during postnatal development. This suggested a functional role of the L5 epitope, or of the molecules carrying it, at both stages. Here, we review the evidence indicating that the carbohydrate may be a marker for cells competent to respond to neural inducing signals and seems itself to be involved in the early response to neural inducing signals. In postnatal cerebellar development, when L5 is found on astrocytes, it participates in the outgrowth of astrocytic processes on extracellular matrix components.

L5 during neural induction in the chick

Around the beginning of gastrulation, cells of the ectoderm are instructed to change their fate and to develop into neural, instead of epidermal tissue. Initial experiments in amniotes by Waddington [42] established the tip of the primitive streak (Hensen's node), as the source of neural inducing signals (see also [11, 29, 36]). One striking finding is that, in the chick embryo, it is not only the embryonic ectoderm that can respond to these signals, but the extraembryonic epiblast, normally fated to give rise only to extraembryonic membranes, can respond to a graft of Hensen's node by producing a complete central nervous system (CNS), expressing markers for all regions. As development proceeds, the competence of the ectoderm to respond to a graft of Hensen's node is lost, first from the extraembryonic *(area opaca)* and then from the embryonic *(area pellucida)* epiblast.

At early stages of chick embryo development (fig 1A–E), L5 immunoreactivity first appears around the time of primitive streak formation in a few scattered cells of the epiblast of the *area pellucida* (fig 1A). During primitive streak elongation, expression of the L5 epitope expands in the epiblast until it covers a broad area, centered around the anterior primitive streak and extending into the inner third of the extraembryonic *area opaca* (fig 1B, C). After stage 4, the definitive primitive streak stage, L5 immunoreactivity becomes gradually restricted, until by stage 6 it is confined to the presumptive neural plate (fig 1D). Thus, at mid- to late primitive streak stages, the expression pattern of L5 matches the region competent to respond to a graft of Hensen's node, and thereafter it matches the area fated to become neural tissue. After neurulation, L5 remains confined to the entire developing nervous system for several days (fig 1E). Therefore, during the time of neural induction, the L5 carbohydrate could be a marker for cells that are competent to respond to neural inducing signals. After formation of the neural plate, it is expressed in the tissue that results from such an induction, the nervous system itself.

When a graft of Hensen's node is placed into an ectopic region, it causes an increase of L5 expression as well as its long-term maintenance in the epiblast surrounding the graft.

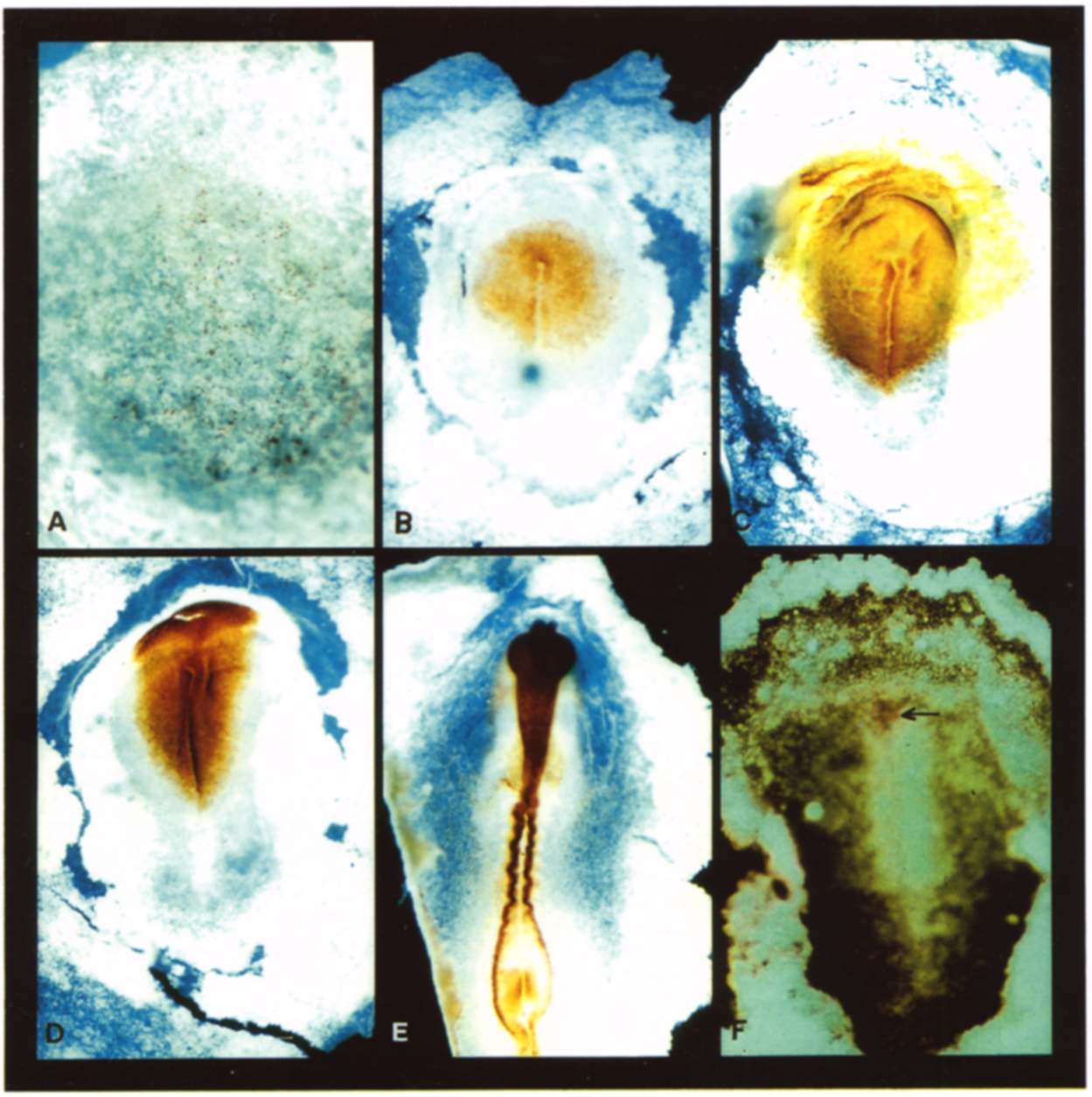

Fig 1. Expression of the L5 epitope and HGF/SF in the chick embryo. **A.** Stage XIII. L5 is first expressed in a few scattered cells in the central epiblast of the *area pellucida*. **B.** Stage 3+. L5 expression surrounding the anterior primitive streak. **C.** Stage 4. L5 immunoreactivity expands as far as the inner third of the *area opaca*. **D.** Stage 6. L5 expression becomes confined to and upregulated in the presumptive neural plate area. **E.** Stage 9−. L5 is expressed in the entire neural tube. **F.** Whole mount *in situ* hybridisation showing the expression of HGF/SF transcripts at stage 3+, in a triangle of cells in the anterior part of Hensen's node (arrow).

If L5 indeed is a marker for competence, this observation predicts that Hensen's node can maintain the competence of the neighbouring epiblast to respond to neural inducing signals. This hypothesis has not yet been tested experimentally.

As part of a search for molecular signals that might be involved in this process, the role of hepatocyte growth factor/scatter factor (HGF/SF) was investigated. When beads coated with HGF/SF or cells secreting it are transplanted into a young chick embryo, ectopic neural plate like structures sometimes form in the epiblast adjacent to the graft [35]. By *in situ* hybridization (fig 1F), expression of chick HGF/SF was found to be localized to Hensen's node at the time when it possesses neural inducing activity, and is downregulated rapidly at precisely the time when it loses such an activity [39].

To address the question of whether HGF/SF is responsible for the maintenance of L5, an *in vitro* assay was developed, in which explants of *area opaca* epiblast are cultured in three-dimensional collagen gels in the presence of soluble factors. When HGF/SF is included in the medium, the explants, which would normally lose L5 expression within a few hours, not only continue to express but also rapidly upregulate the expression of L5. In long-term cultures HGF/SF promotes the differentiation of cells with neuronal morphology and expression of neural-specific markers such as neurofilament proteins [39]. These experiments led to the proposal that HGF/SF might be involved in the early

steps of neural induction by maintaining L5 expression – and possibly neural competence – in the presumptive neural plate region.

To address whether the L5 epitope itself plays a role in early neural development, Hensen's node was grafted into host embryos together with hybridoma cells secreting the L5 monoclonal antibody or with control hybridoma cells. In the presence of control cells, the node induced ectopic neural structures, as expected. In the presence of L5 hybridoma cells, however, the formation of newly induced neural tissue was completely inhibited. These data indicate that either the L5 epitope itself, or the molecules carrying it, play an important role in neural induction and suggest that it might be involved in the early response of the ectodermal cells to the inducing signals.

In summary, the data described above suggest that in the early embryo the L5 carbohydrate labels the area competent to respond to neural inducing signals. Hensen's node and HGF/SF, expressed transiently in the node, both maintain L5 expression (and presumably neural competence) in the epiblast. In addition, L5 seems to be involved in the early response of cells to the inducing signal. After neural induction, L5 is expressed in the structures that result from this induction, the entire nervous system, but its role at these stages is still unknown.

As mentioned above, the L5 antigenic determinant was identified to be closely related to Le^x, a carbohydrate structure, which has itself been implicated in cell-cell interactions (*eg* the compaction of mouse embryos [8, 12, 17, 30]). These observations suggest that the L5 epitope might mediate cellular interactions between the cells that receive the neural inducing signals. This could occur either *via* carbohydrate-carbohydrate interactions, as was proposed for Le^x [6, 12], or could involve a complementary lectin-like ligand on the cell surface. The exact molecular mechanism, however, remains unclear. There have been two recent reports [15, 27] that transgenic mice lacking N-acetylglucosaminyltransferase I, the enzyme initiating the synthesis of complex-type carbohydrates, show defects in neuroepithelial development and in the closure of the neural tube. This underlines the possible functional importance of such complex carbohydrate moieties during early neural development.

Carbohydrates expressed on molecules belonging to the L2/HNK-1 family mediate cell interactions

The L5 antibody was generated against murine brain glycoproteins of the L2/HNK-1 family of cell adhesion molecules [33] and belongs to a series of monoclonal antibodies, which have been designated L2/HNK-1 + [19, 33], L3 [20] and L4 [7]. All of them, including the L5 antibody, identify distinct cell surface oligosaccharides present on different cell adhesion molecules [3, 4, 33, 39] and the antibodies themselves were used to address the functional role of these carbohydrates in cellular interactions.

The L2/HNK-1 epitope is expressed by glycolipids and several cell recognition molecules in the peripheral and central nervous system (as well as other non-neural cells). This epitope was shown to mediate the outgrowth of processes in astrocytes and neurons [21], the binding of neuronal cells to the extracellular matrix component laminin [13], neurite outgrowth of motor but not sensory neurons [25], migration of neural crest cells [2] and finally to be a ligand for L- and P-selectin [28].

The L3 and L4 antibodies apparently recognize very closely related N-linked oligomannosidic structures and are coexpressed on several cell adhesion molecules [7, 34]. Despite their close structural relation they seem to react with different epitopes and the corresponding carbohydrates seem to perform distinct functional tasks. While the L4 carbohydrate mediates neuron-neuron and neuron-astrocyte adhesion, the L3 antibody interferes with neuron-neuron binding only [7]. Moreover, oligomannosidic L3 glycans are necessary for the interaction of two calcium-independent cell adhesion molecules, L1 (Ng-CAM) and NCAM, on the same cell surface, thereby supporting neurite outgrowth [14].

L5 in the postnatal development of the mouse cerebellum

During development of the mouse cerebellum, L5 expression peaks at the end of the first postnatal week, which coincides with a period when important morphogenetic events take place, such as the formation of radial Bergmann glia processes and granule cell migration along these processes. The L5 epitope is expressed in the molecular and in the external and internal granular layers of the developing cerebellum. Frequently, radial stripes of immunoreactivity can be observed in the external granular layer, which are reminiscent of the radial processes of Bergmann glia (A Streit and M Schachner, unpublished results). Astrochondin, the only L5-positive molecule on astrocytes, is present on astrocyte cell surfaces along Bergmann glia fibers and their endfeet abutting to the *pia mater* as well as on the astrocyte endfeet that contact blood vessels [38]. *In vitro,* the L5 epitope is also predominantly expressed by a subpopulation of immature (vimentin-positive) and mature (glial-fibrillary-acidic-protein-positive) astrocytes (fig 2A–C). After several days in culture, cerebellar neurons also become weakly L5 positive [37].

Similar results have been obtained in some studies investigating the presence of the Le^x determinant, which is related to L5, showing its predominant expression on astrocytes [22, 32]. In different species, however, the cell-type-specificity of the Le^x epitope seems to vary [1, 5, 23, 24, 41]; even within the same organism, conflicting results have been reported. For example, in humans, McCarthy *et al* [26] reported Le^x expression on astrocytes in the fetal and adult central nervous system, whereas other studies showed its presence either in the adult only [10] or on fetal neurons as well [9, 24]. Additionally, some of these studies suffer from the lack of use of cell type specific markers and the fact that different antibodies with distinct fine specificities were used for detection, which complicates the interpretation of the data.

In vitro assays provided insight into the possible functional role of the L5 epitope during cerebellar development and suggested that the L5 epitope is involved in the formation of astrocytic processes. In explant cultures of postnatal cerebellum, L5 antibodies inhibited the outgrowth of astrocyte processes, whereas they had no effect on neurite outgrowth or neuronal migration (fig 2D–I). Similar results were obtained for the L5 positive proteoglycan astrochondrin. Astrochondrin itself was shown to bind to the extracellular matrix components laminin and collagen type IV, on which antibodies against it inhibit the formation of processes by astrocytes. It was therefore suggested that astrochondrin and the L5 epitope can support the interaction of astrocytes with extracellular matrix components thereby leading to the outgrowth of processes. This function can be closely correlated with the spatial and temporal expression

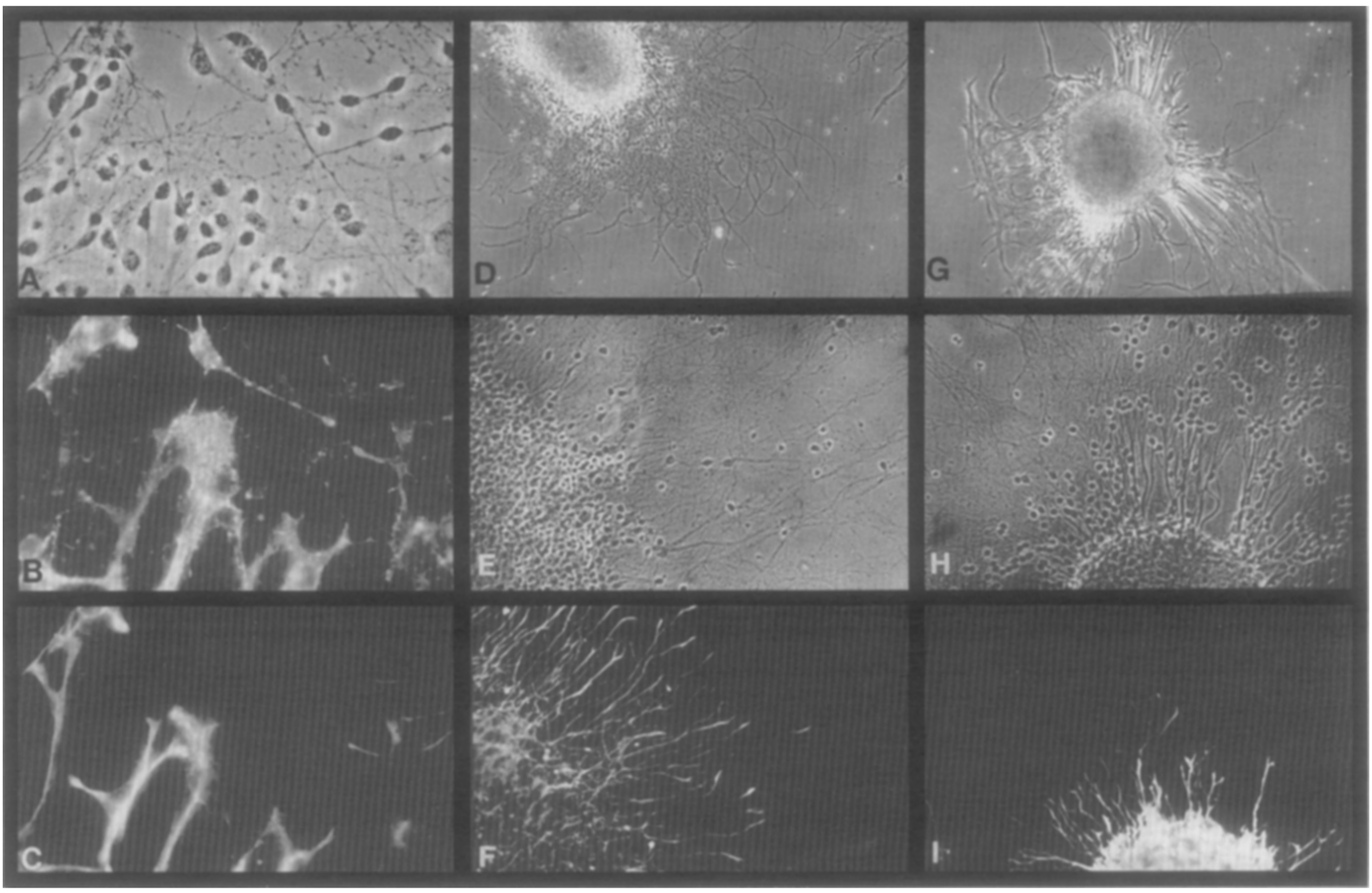

Fig 2. L5 expression on cerebellar astrocytes and inhibition of astrocyte process outgrowth by L5 antibodies. **A–C.** Double immunofluorescence labelling of cerebellar cell cultures from 6-day-old mice with L5 antibody **(B)** and glial fibrillary acidic protein **(C)** after 3 days in culture. **A.** Phase contrast views for **B** and **C.** L5 is expressed by GFAP positive astrocytes. **D–I.** Cerebellar explants from 6-day-old mice were cultured in the presence of Fab fragments of polyclonal antibodies to mouse liver membranes **(D–F)** or of monovalent IgM fragments of the L5 antibody **(G–H). D, G.** Representative explants after 2 days in culture. After 3 days *in vitro,* explants were stained by indirect immunofluorescence with antibodies to GFAP **(E, F, H, I)** to label astrocytes. L5 antibody inhibits the formation of astrocytic processes. **E, H.** Phase contrast views for **F** and **I,** respectively.

of the L5 epitope, just at the time when Bergmann glia cells send out newly-generated processes in a radial way to make contact with the extracellular matrix of meningeal cells. The Bergmann glia fibers provide a scaffold along which granule cells migrate from the external to the internal granular layer. Antibodies to astrochondrin prevent this migration of granule cells. Since, however, neither these antibodies nor the L5 antibody interfere with neuron-astrocyte binding in short term cell adhesion assays or with neurite outgrowth or migration of neurons on astrocyte monolayer cultures, this observation was interpreted as an indirect effect due to the disturbance of astrocyte process formation.

The outgrowth of astrocyte processes takes place not only during normal development, but also after injury of the central nervous system [16]. Astrocytic scar formation is accompanied by extensive process outgrowth; we do not know whether expression of the L5 epitope is upregulated under these circumstances. A recent study, however, reported that reactive astrocytes strongly re-express SSEA-1, a closely related (if not identical) carbohydrate, after injury of the optic nerve. Furthermore, immortalized astrocytes show upregulated expression of CD15, which is also closely related or identical to L5, at contact sites and particularly in their processes. Both observations support the idea that the L5 epitope indeed might be involved in the outgrowth of astrocyte processes.

Conclusion

In summary, the L5 epitope, a complex carbohydrate closely related to the structures Le^x, SSEA-1 and CD15, is expressed at various stages of neural development from the very earliest steps of neural induction to postnatal development of the mouse cerebellum. In those systems where this has been studied, it appears to be involved directly in mediating cell interactions, including the response to neural inducing signals and the outgrowth of processes in cerebellar astrocytes. Further research will be required to elucidate its function at intermediate stages of development.

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