N-cadherin protein distribution in normal embryos and in embryos carrying mutations in the homeobox gene Hoxa-4

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ABSTRACT N-cadherin is a calcium-dependent adhesion molecule with a potential role in a variety of morphogenetic events. Although a dynamic pattern of expression in the mouse embryo has been suggested by in situ hybridization analysis, to date there has been no report of N-cadherin protein expression. In this immunohistochemical study we surveyed N-cadherin protein expression in the mid-late gestation mouse embryo utilizing a recently characterized monoclonal antibody. We found N-cadherin expression in a wide array of tissues, including the brain, the eye, various cranial ganglia, the spinal cord, spinal ganglia, somites, vertebral and limb cartilage and perichondria, the developing lung and kidney, the enteric nervous system, and germ cells. These results suggest that N-cadherin protein expression, as in the chick embryo, correlates with the segregation of cells and with organogenesis. As cadherins have been proposed as targets of vertebrate Hox genes, we also examined N-cadherin expression in two lines of Hoxa-4 mutant mice. We did not observe any alterations in N-cadherin expression in either Hoxa-4 null embryos or in transgenic embryos that overexpress Hoxa-4 in the mesenchyme of the gut. However, the partial overlap in expression between Hox genes and N-cadherin, and the likelihood of redundancy in the regulation of target genes, leaves open the possibility that cadherins are direct or indirect targets of Hox genes during mouse embryogenesis.

KEY WORDS: N-cadherin, immunohistochemistry, embryogenesis, Hoxa-4

Introduction

Cadherins are calcium-dependent adhesion molecules that are expressed in a wide array of tissues during embryogenesis and in the adult (Takeichi, 1990). Each member of the expanding cadherin superfamily exhibits a unique pattern of expression during development, although there is significant overlap in expression between certain cadherins (Takeichi, 1988, 1991; Ranscht, 1994). Cadherin-bearing cells have been demonstrated to adhere preferentially to adjacent cells bearing the same cadherin in vitro (Kemler, 1992), and perturbation of cadherin expression or function in vivo has been demonstrated to affect embryonic development (Kintner, 1992; Holt et al., 1994; Larue et al., 1994). Given the association of cadherin expression with various developmental processes, it has been proposed that cadherins are involved in the separation of the germ layers during gastrulation (Hatta and Takeichi, 1986), and in the segregation, rearrangement, and assembly of cells during organogenesis (Hatta et al., 1987). In addition, disruption of cadherin function has been associated with both tumorogenesis (Birchmeier et al., 1993), and increased metastatic potential (Viemnickx et al., 1991). Most recently, inhibition of cadherin function has been linked with inflammatory bowel disease in mice (Hermiston and Gordon, 1995).

N-cadherin is a well-studied member of the cadherin superfamily. Initially identified as an adhesion molecule in the mouse and chicken central nervous systems (CNS) (Hatta et al., 1985), its expression has been examined by in situ hybridization and immunofluorescence in the developing chick embryo (Hatta and Takeichi, 1986; Hatta et al., 1987; Inizuka et al., 1991b), and by in situ hybridization in the mouse embryo, with particular emphasis on its expression in the brain (Redies and Takeichi, 1993). In the chick embryo, N-cadherin protein was found to be widely expressed throughout embryonic development; prominent sites of expression include cardiac and skeletal muscle, somites, the urogenital system, the central and peripheral nervous systems, and various neural crest derivatives and mesenchymal cells (Hatta et al., 1987). It has been proposed that N-cadherin is involved in promoting developmentally scheduled associations between different cell types (Hatta et al., 1987), and functional studies in the chick embryo have identified a role for N-cadherin

Abbreviations used in this paper: CNS, central nervous system; PGC, primordial germ cell; DAB, 3,3'-diaminobenzidine.

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in various aspects of neurogenesis, including neural cell adhesion (Miyatani et al., 1989) and axon outgrowth (Neugebauer et al., 1988; Bixby and Zhang, 1990). *In vitro* studies have indicated that N-cadherin is involved in myoblast fusion (Knudsen et al., 1990) and in cardiac myocyte interaction (Peralta Soler and Knudsen, 1994).

Transcriptional activators of cadherins have not yet been identified, but several investigators have proposed that Hox genes are good candidates for such a role. Gene targeting experiments in which Hox genes have been altered or deleted have begun to clarify their roles in pattern formation and organogenesis. Mice carrying mutations in a single Hox gene typically exhibit homeotic transformations of vertebrae at the gene's anterior limit of expression (reviewed in Manak and Scott, 1994), as well as defects in limbs (Davis et al., 1995), neural crest derivatives (Manley and Capecchi, 1995), and other tissues such as thymus (Manley and Capecchi, 1995), kidney (Davis et al., 1995), and vas deferens (Hsieh-Li et al., 1995). Mice lacking two or three paralogous Hox genes generally have more severe phenotypes (Horan et al., 1995). *In vitro* experiments have demonstrated that Hox genes can bind to homeodomain binding sites in the regulatory regions of adhesion molecule genes (Goomer et al., 1994), and the calcium-dependent and -independent adhesion molecules have been proposed as likely targets of Hox genes since they can provide a link between pattern formation and morphogenesis (Edelman, 1988).
N-cadherin protein expression has been less well studied in mammalian embryos, due largely to the unavailability of adequate antisera. We have undertaken an immunohistochemical analysis of N-cadherin expression in mouse embryos, using a recently developed monoclonal antibody that has been demonstrated to detect N-cadherin in cultured human fibroblasts (Knudsen et al., 1995). In addition to characterizing its expression in wild-type embryos, the reports implicating Hox genes as direct regulators of cadherins prompted us to examine N-cadherin expression in two established lines of Hox mutant mice, in an attempt to determine if the phenotypes of these mice could be attributed, in whole or in part, to altered cadherin expression. We discuss these results in the context of the developmental events that might be mediated by N-cadherin in mammalian embryos.

**Results**

**Expression at E9.5-E10.5**

The earliest embryos examined for N-cadherin expression were at day 9.5. At this time, N-cadherin was found in the developing heart, with strong expression in the myocardium of the atrial and ventricular chambers and in the truncus arteriosus (Fig. 1A,B). The endocardium was not stained. Myocardial expression was also seen at E12.5 (not shown). At E9.5, transverse sections were examined for expression in the neural tube and somites. Staining was apparent in the neural tube and in all cells of the somites, with particularly strong expression at the lumenal side of the somitic epithelial vesicles (Fig. 1C). Figure 1D shows a control embryo at E12.5 in which the primary antibody was omitted. There is little or no staining seen.
Expression at E12.5-E15.5

Neuroectoderm

At day 12.5, N-cadherin was prominently expressed in the developing eye (Fig. 2B). In particular, expression was localized to the lens and the neuroretina, with high level expression in the innermost layer, where the first postmitotic neurons are produced, and in the outermost layer. The inner nuclear layer appeared to be uniformly stained at a low level. N-cadherin was also widely expressed in the brain at E12.5 (Fig. 2A,C,D), in a pattern similar to that found in the chick embryo. Sites of expression include the cortex, the diencephalon, the roof of the midbrain, the pons, and the intraventricular portion of the cerebellum (Fig. 2A,C). Several cranial ganglia were also positive, including the trigeminal ganglion, the superior cervical ganglion, and the facial, glossopharyngeal, and vagus nerves (Fig. 2A). The posterior commissure was also stained, and Figure 2D shows a high magnification view of N-cadherin along the commissural fibers. Figure 3A shows a transverse section through the thoracic region of an embryo at E12.5, with prominent expression in the dorsal spinal cord, and in the floor plate, spinal ganglia, and nerve roots as well. A high magnification view of the ventral half of the spinal cord is shown in Figure 3B, highlighting the expression in the floor plate and in the marginal layer. Positively stained spinal nerves and ganglia are shown in a parasagittal section in Figure 3C. The patterns of N-cadherin expression throughout the CNS are consistent with its previously reported distribution in the chick embryo (Hatta et al., 1987; Inizuka et al., 1991b).

Enteric nervous system

N-cadherin exhibited a precise pattern of expression in the developing gut. In the umbilical gut, at E12.5, expression was restricted to individual cells that are likely enteric neuroblasts (Fig. 4A). A circular ganglion was clearly labeled. At E15.5, staining of a longitudinal section of the gut revealed expression in the myenteric plexus, sandwiched between unstained serosa and unstained smooth muscle cells (Fig. 4C). Adjacent to the smooth muscle layer was the lightly stained submucosa. The mucosa was unstained. A higher magnification view of the myenteric plexus shows that a few of the intensely stained cells had prominent nucleoli, while others were stained along what appeared to be axonal cytoplasm (Fig. 4D,E). These features are characteristic of neuronal cells, and support the conclusion that N-cadherin is highly expressed in the myenteric plexus. A longitudinal section through the stomach at E15.5 revealed a similar pattern of expression, with staining in the myenteric plexus and submucosal mesenchyme (not shown). This expression profile, with intense staining in the enteric ganglia and lighter staining in adjacent mesenchyme was consistent along the entire axis of the gut.

Mesoderm

N-cadherin expression was found in vertebral bodies, in perichondria, and in neural arches at E15.5 (Fig. 5A,B,C), but not at E12.5 (not shown). In the developing limbs, N-cadherin was expressed at a high level in the perichondria (Fig. 5D,E) and in the surrounding mesenchyme (not shown). In the limb cartilage, staining was seen in the hypertrophic zone, but not in the adjacent proliferative zone (Fig. 5D,E). Figure 5F shows staining in the mesenchyme surrounding a rib.

N-cadherin expression was also found in the developing urogenital complex. At E14.5, light staining was seen in the tubules of the metanephros and in groups of cells that were in the process of condensing into tubules (Fig. 6A). In the embryonic testis at E14.5, expression was seen in groups of spermatogenic cells that were clustered in the lumens of the newly formed seminiferous tubules (Fig. 6B). N-cadherin did not appear to be expressed in the pre-Sertoli cells along the basal laminae of the tubules.

In the developing lung, at E12.5, N-cadherin was found to be uniformly distributed in the mesenchyme, and was absent from the epithelia surrounding the bronchii (Fig. 7A,B). In the same section
N-cadherin expression in Hoxa-4 mutant mice

We have previously generated mice lacking the Hoxa-4 gene, which exhibit homeotic transformations of cervical vertebrae (Horan et al., 1994). Given the partial overlap in expression between Hoxa-4 and N-cadherin – including the perichondria of the developing vertebrae, the dorsal neural tube and floor plate, the spinal ganglia and nerve roots, and the mesenchyme in the developing lung and gut (Galliot et al., 1989; Behringer et al., 1993) – we decided to examine N-cadherin expression in the Hoxa-4-deficient mice. Figure 8A shows a Hoxa-4 null embryo at E12.5, with expression apparent in the spinal ganglia, nerve roots, heart, and brain. Staining of serial parasagittal sections throughout this embryo revealed N-cadherin expression in a pattern essentially identical to that of wild-type embryos. Expression in the gut at E12.5 was also unchanged (Fig. 4B – compare to Fig. 4A). At E14.5, N-cadherin was found in the perichondria surrounding the ribs and in the lung (Fig. 8B). Overall, at E12.5 and E14.5 we observed no obvious differences in N-cadherin expression between wild-type and Hoxa-4 null embryos.

As an alternative approach to determining whether N-cadherin expression is regulated by Hoxa-4 in vivo, we utilized another line of Hoxa-4 mutant mice, 1975-2, which is a transgenic line that overexpresses Hoxa-4 in the CNS, lung, and especially in the mesenchyme of the gut (Wolgemuth et al., 1989). These mice develop lethal megacolon due to hypoganglionosis in the distal colon (Wolgemuth et al., 1989; Tennyson et al., 1993). This phenotype may result from a defective interaction between the precursors of enteric neurons and the smooth muscle cells of the gut (Tennyson et al., 1993). Given the strong expression of N-cadherin in enteric neurons (Fig. 4) and the co-expression of N-cadherin and Hoxa-4 in the gut mesenchyme (Galliot et al., 1989; Behringer et al., 1993), we were particularly interested in determining if N-cadherin expression was affected by overexpression of
Fig. 5. N-cadherin in prevertebral and limb cartilage at E15.5. (A) Transverse section of a vertebral body showing cell surface staining. (B) Transverse section of a neural arch, showing cell surface staining. (C) Transverse section through the hindlimb, with staining in the perichondrium and in the hypertrophic cells of the differentiating cartilage. (D) Transverse section through the hindlimb, with staining in the perichondrium and the hypertrophic zone of the cartilage, but not in the proliferative zone. (E) Transverse section through the hindlimb, with staining in the perichondrium and on the surface of the hypertrophic cells. (F) High magnification view of a transverse section through a rib, showing staining in the surrounding mesenchyme. HZ, hypertrophic zone; PZ, proliferative zone; P, perichondrium; R, rib.

Hoxa-4 in the gut. As shown in Figure 8D and E (compare to Fig. 4), N-cadherin expression was not obviously affected in the transgenic mice, at either the most caudal level of the hindgut or at a more rostral level (at the level of the kidney), suggesting that the hypoganglionosis in these mice cannot be explained by elevated N-cadherin expression.

Discussion

In this immunohistochemical study we have identified the pattern of N-cadherin protein expression in the mid-late gestation mouse embryo. As in the chick, expression corresponds to sites of epithelial cell arrangement and of segregation of cells through differential cell-cell adhesion (Hatta et al., 1987).

At the earliest time examined, N-cadherin was found to be highly expressed in the neural tube, somites, and myocardium of the developing heart. Previous observations on the role of N-cadherin in the function of chick embryonic cardiomyocytes have established that N-cadherin is an essential component of intercellular junctions and is required for the spontaneous contraction of these cells in vitro (Peralta Soler and Knudsen, 1994). Moreover, it was found that cardiomyocytes treated with an anti-N-cadherin antiserum had disorganized myofibrils, suggesting the involvement of N-cadherin not only in myocyte adhesion and electrical coupling, but in myofibrillogenesis as well. The recent targeted disruption of the N-cadherin gene in mice resulted in embryonic lethality by E10 due to abnormal development of the myocardium of the primitive heart, establishing that N-cadherin is essential for heart development in vivo (Radice et al., 1997). Defects were also observed in neural tube and somite development, consistent with the pattern of expression of N-cadherin in the E9-E10 mouse embryo.
Fig. 6. N-cadherin in the urogenital system at E14.5. (A) Transverse section through the kidney, showing staining in condensing tubules (arrows). (B) Transverse section through the testis, with staining in the spermatogenic cells (arrowheads), but not in the pre-Sertoli cells (arrows).

Although we did not carry out a detailed analysis, our observations of N-cadherin protein expression in the embryonic brain are consistent with the results reported at the mRNA level (Redies and Takeichi, 1993). Our findings of N-cadherin in the dorsal spinal cord, and also in the floor plate, spinal ganglia, and nerve roots, are in agreement with results reported for the chick (Inizuka et al., 1991a). In addition, the strong expression in the lens and in both the inner and outer plexiform layers of the neuroretina are consistent with immunofluorescent studies of the chicken eye (Matsunaga et al., 1988; Inizuka et al., 1991a).

Expression of N-cadherin protein in tissues of mesodermal origin was detected in prevertebrae and perichondria, lung mesenchyme, and in condensing metanephric tubules, suggesting again a role in sorting out different cell layers or populations. Functional tests of the role of N-cadherin in chick limb chondrogenesis have established that it is required for the aggregation of mesenchymal cells and for overt differentiation (Oberlender and Tuan, 1994). The present results suggest that N-cadherin may have a similar role in chondrogenesis in the mouse embryo.

The detection of N-cadherin in spermatogenic cells at E14.5 is suggestive with regard to a role in germ cell-germ cell adhesion during the migratory and postmigratory phases of gametogenesis in the mouse. Although N-cadherin expression had been noted in the embryonic germ cells of the chick (Hatta et al., 1987), to our knowledge this is the first report of cadherin expression in mouse embryonic germ cells. Recent studies (Gomperts et al., 1994) of mouse primordial germ cell (PGC) migration using confocal microscopy have established that PGCs migrate in groups rather than as single cells, by first extending long processes and then by aggregating. It has been proposed that adhesion may promote germ cell migration and accumulation in the genital ridge and may have a role in inhibiting the migratory potential of these cells once they reach the gonad. Although we did not examine migrating primordial germ cells for N-cadherin expression, the presence of N-cadherin protein in germ cell clusters in the gonad raises the possibility that it has such a role. Moreover, reports of N-cadherin mRNA in Sertoli cells, and in meiotic and postmeiotic germ cells in the postnatal mouse testis (Byers and Blaschuk, 1993), and N-cadherin protein in Sertoli cells and germ cells in the postnatal rat testis (Newton et al., 1993), suggest that N-cadherin may be an important factor regulating germ cell adhesiveness throughout the development of the germ line. The recent success in producing mature rat spermatozoa by transplanting rat spermatogonial stem cells into mouse testis (Clouthier et al., 1996), may in part be due to the apparent conservation of N-cadherin-mediated germ cell-Sertoli cell adhesion between rat and mouse.

Our observations of N-cadherin expression in the precursors of enteric neurons, in the myenteric plexus, and in the submucosal mesenchyme confirm results from studies of the chick (Hatta et al., 1987), and suggest that N-cadherin has a role in promoting cell-cell interactions in the developing enteric nervous system. N-cadherin may be involved in mediating the migration of the enteric neuroblasts...
though the surrounding mesenchyme or in promoting interneuronal adhesion as the neuroblasts coalesce to form ganglia.

Although there was no apparent effect of Hoxa-4 overexpression in the gut on N-cadherin expression, it is possible that N-cadherin expression is affected in other mouse models of hypoganglionosis/aganglionosis. Recently, the defect in Dominant megacolon mutant mice, which exhibit intestinal aganglionosis, was demonstrated to be due to an abnormal microenvironment (Kapur et al., 1996). In addition, the defect in mice homozygous for the piebald lethal mutation, which have a deletion of endothelin receptor-B, and which exhibit intestinal aganglionosis, was also found not to be completely neuroblast autonomous (Kapur et al., 1995). An alteration in N-cadherin-mediated cell-cell adhesion and/or signaling is one way in which these mutant phenotypes might be generated.

Our results suggest that N-cadherin is involved in a variety of morphogenetic processes during mouse embryogenesis. In addition to the heart, neural tube, and somite defects observed in the knockout mice (Radice et al., 1997), particular areas of interest include, but are not limited to, neurogenesis in the retina, aggregation and differentiation of chondrocytes in the vertebral and limbs, migration and differentiation of enteric neurons, and germ cell aggregation and migration. Conditional knockouts and in vitro experiments using reagents that inhibit N-cadherin function will be required to identify its full range of functions in embryonic and adult tissues.

It remains an open question as to whether N-cadherin expression is regulated by HOX proteins. Although we observed no alterations in N-cadherin expression in either of our two Hox mutant lines, paralogous Hox genes may maintain expression of target genes. Studies of the expression of N-cadherin and other adhesion molecules in mice lacking two or more paralogous Hox genes may help to resolve this question. Recent experiments in which the HOXA-13 protein was misexpressed in chick limb buds, resulting in limb mesenchymal cells with altered adhesive properties, suggest that under certain conditions a link between HOX proteins and adhesion can be demonstrated (Yokouchi et al., 1995). Given the partial overlap of expression between HOX proteins and several adhesion molecules, including N-cadherin, further experiments in which HOX proteins are ectopically expressed may confirm such a link.

Materials and Methods

Source of tissues

Normal embryos were obtained from timed matings of Swiss Webster mice (Charles River, Wilmington, DE, USA). The day of detection of the vaginal plug was considered embryonic day 0.5. Hoxa-4 null embryos were derived from the mating of Hoxa-4 null adults, whose genotypes were confirmed by Southern blot analysis of tail DNA according to standard procedures (Horan et al., 1994). Transgenic embryos in which Hoxa-4 is expressed at elevated levels were derived from the mating of heterozygous 1975-2 males with C57BL6J females, and were genotyped by Southern blot analysis of placental DNA according to standard procedures (Wolgemuth et al., 1989).

Immunohistochemistry

Embryos were fixed overnight in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After rinsing in phosphate-buffered...
saline (PBS) the embryos were dehydrated and embedded in paraffin according to standard procedures. Sections (6 μM) were cut and mounted on Superfrost slides (Fisher). The slides were deparaffinized in Histoclear and dehydrated through an alcohol series, and were then boiled in 0.01 M citrate buffer, pH 6.0, in a microwave for 10 min (Shi et al., 1991), allowed to cool, and washed in distilled water. Following a 20 min incubation in methanol containing 0.3% hydrogen peroxide, the slides were washed for 20 min in PBS and then incubated for 1 h in blocking solution [10 ml PBS containing 1% bovine serum albumin (Fraction V BSA, Calbiochem) and 3 drops of normal horse serum]. The slides were then incubated overnight in a humidified chamber in the blocking solution containing a 1:10 dilution of a monoclonal antibody that recognizes the intracellular domain of N-cadherin (13A9 conditioned medium; see Knudsen et al., 1995). The excess antisera was blotted off, the slides were washed for 30 min in PBS, and they were incubated for 1 h with an anti-mouse biotinylated antibody from the Vectastain ABC kit (Vector Laboratories; 10 ml PBS containing 1% BSA and one drop of the secondary antibody). Excess antisera was blotted off, the slides were washed for 30 min in PBS, and they were incubated for 2 h with the Vectastain ABC reagent in PBS according to the manufacturer's instructions. The slides were washed in PBS for 30 min, stained with DAB, and counterstained with hematoxylin according to the manufacturer's instructions. They were viewed under bright-field optics on Leitz and Wild Heerbrugg photomicroscopes, and photographs were taken using FujiColor 100 film.

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