Site-restricted Expression of Cytotactin during Development of the Chicken Embryo

Kathryn L. Crossin, Stanley Hoffman, Martin Grumet, Jean-Paul Thiery,* and Gerald M. Edelman

The Rockefeller University, New York, New York 10021; and *Institut d'Embryologie du Centre National de la Recherche Scientifique et du Collège de France, 94130 Nogent-sur-Marne, France

Abstract. The sequential appearance of the extracellular matrix (ECM) protein, cytotactin, was examined during development of the chicken embryo by immunohistochemical techniques. Although cytotactin was identified as a molecule that mediates glia–neuron interactions, preliminary immunohistochemical localization of the molecule suggested that it was an ECM protein with a widespread but nonetheless more restricted distribution than either fibronectin or laminin. In the present study, it was found that cytotactin is first present in the gastrulating chicken embryo. It appears later in the basement membrane of the developing neural tube and notochord in a temporal sequence beginning in the cephalic regions and proceeding caudally. Between 2 and 3 d of development, the molecule is present at high levels in the early neural crest pathways (surrounding the neural tube and somites) but, in contrast to fibronectin and laminin, is not found in the lateral plate mesoderm or ectoderm. At later times, cytotactin is expressed extensively in the central nervous system, in lesser amounts in the peripheral nervous system, and in a number of non-neural sites, most prominently in all smooth muscles and in basement membranes of lung and kidney. Cytotactin appears in adult tissues with distributions that are similar to those seen in embryonic tissues. The findings raise the possibility that certain ECM proteins contribute to pattern formation in embryogenesis as a result of their restricted expression in a spatiotemporally regulated fashion at some sites but not at others.

During development, collectives of cells that have different histories move together and influence each other, inducing specific cytodifferentiation events as a result of their mutual interaction (3, 33, 40). Such embryonic induction results in morphogenetic patterning in the early embryo as well as in the development of more complex tissues and organs. Morphoregulation, or the establishment of pattern, is therefore highly dependent upon both the interactions among groups of cells and their subsequent movement. It has been proposed that the coordinate spatiotemporal expression of cell surfaces of cell adhesion molecules (CAMs)1 and substrate adhesion molecules (SAMs) provides a means of regulation of these processes (10–13). This proposal implies that CAMs and SAMs appear in defined sites at characteristic times during development.

A number of CAMs have been identified (2, 7, 16, 17, 22–25), each of which appears in a distinct sequence during embryogenesis and histogenesis. N-CAM, the neural cell adhesion molecule (23), and L-CAM, the liver cell adhesion molecule (16), are both present in the chicken blastoderm (15) and undergo dramatic redistributions at the time of primary neural induction (15, 41, 42). These two CAMs are present in very early embryos, are both expressed on derivatives of all three germ layers during development, and are therefore designated primary CAMs. In contrast, the neuron–glia cell adhesion molecule (Ng-CAM), which is involved in neuron–glia (17) and neuron–neuron (18) adhesion in vitro, appears later in development on neuroectodermal derivatives only (8, 43); it is designated a secondary CAM. The distributions of each of these CAMs have been correlated with major morphological events during embryogenesis (6, 7, 15, 22, 25, 31, 39, 41) and histogenesis (4–6, 8, 14, 29, 32, 35–38, 42, 43), and their expression occurs in restricted but contiguous regions in a classical fate map (48) of the avian blastoderm (12, 13, 43).

The existence of such distributions implies developmental regulation of the expression of these molecules. A major means of regulation of cell adhesion occurs through modulation of CAMs at the cell surface by alterations in the amount or prevalence, distribution, or chemistry of these cell surface proteins (9). A striking example of modulation related to morphogenesis is seen as N-CAM–containing neural crest cells begin to migrate. These cells lose N-CAM and migrate through fibronectin-rich regions, then reexpress N-CAM at sites at which they will differentiate (44). Thus coordinate expression of both a CAM (N-CAM) and a SAM (fibronectin) is correlated first with movement and later with differentiation events. The modulation of CAM expression, the restricted CAM distributions within the fate map, and the known function of CAMs all strongly implicate these molecules as

1. Abbreviations used in this paper: CAM, cell adhesion molecule; CNS, central nervous system; N-CAM, neural CAM; L-CAM, liver CAM; Ng-CAM, neuron–glia CAM; P/NaCl, phosphate-buffered saline; PNS, peripheral nervous system; SAM, substrate adhesion molecule; ECM, extracellular matrix.
regulators of morphogenesis, and suggest that their expression must be correlated with that of SAMs during particular morphogenetic events.

These observations indicate that the temporal and spatial expression of certain surface molecules follows a sequence in development; at any time, this sequence is seen at some regions of the embryo and not at others, i.e., it is site-restricted. It is of particular importance to determine whether certain SAMs, like CAMs, appear in definite sequences that are site-restricted. We have recently identified and isolated a new extracellular matrix (ECM) molecule, cytotactin (20), and have shown that it is involved in glia–neuron adhesion. Cytotactin was initially isolated as one of the proteins that carried a carbohydrate determinant recognized by monoclonal antibody NC-1 (20). NC-1 is a monoclonal antibody raised against quail ciliary ganglia that also recognizes determinants expressed on migrating neural crest cells (47) as well as N-CAM and Ng-CAM (19). Cytotactin appears as three closely related polypeptides of M, 220,000, 200,000, and 190,000 on SDS polyacrylamide gels under reducing conditions; under non-reducing conditions it barely enters a 6% gel, which suggests that the native polypeptides form polymers that are disulfide cross-linked. The molecule is synthesized by glia as well as by cells isolated from kidney, lung, and smooth muscle. It was not seen in striated or cardiac muscle and appeared to be less widespread than the ubiquitous ECM molecules fibronectin and laminin. These findings raised the possibility that cytotactin might, like the CAMs, be restricted to certain specific regions in a classical fate map.

With this in mind, we have now examined the appearance of cytotactin during embryogenesis and have compared its localization with that of fibronectin and laminin. In addition, we have assessed whether the expression of this molecule is correlated with paths of cell migration or with other salient morphogenetic features, implying various related functions in different tissues. The results of this study indicate that cytotactin is a matrix protein with a sharply restricted pattern of expression. They raise the possibility that cytotactin and other such proteins may help to guide morphogenetic movements and subsequent developmental pattern formation by providing a temporally and spatially modulated extracellular environment for interacting groups of cells.

Materials and Methods

Preparation of Tissues

White Leghorn chicken embryos were staged according to the number of somites or according to Hamburger and Hamilton (21) for later stages. Embryos were fixed in 2.5% paraformaldehyde/0.02% glutaraldehyde in 100 mM phosphate buffer (pH 7.3) (P) at room temperature for 10–60 min depending on the age of the embryo. After quenching with 0.1 M glycine in phosphate-buffered saline (P/NaCl), the embryos were infiltrated with 30% sucrose in P/NaCl at 4°C. Very early embryos were first embedded in 1% low-melting-point agarose (Bethyl Research Laboratories, Bethesda, MD) in 18% sucrose/P/NaCl to facilitate handling and orientation. The embryos were mounted in OCT compound (Lab-Tek, Naperville, IL) on dry ice; 10-µm cryostat sections (IEC CTF microtome-cryostat) were attached to poly-L-lysine-coated slides.

Immunofluorescence

Indirect immunofluorescent labeling was done as described (6). Affinity-purified anti-laminin antibodies were purchased from E. Y. Laboratories, Inc. (San Mateo, CA) and anti-fibronectin antibodies were prepared as previously described (30). Preparation of rabbit anti-cytotactin routinely used in staining experiments is described below. Slides were mounted in 90% glycerol/P/NaCl/0.1% p-phenylenediamine to prevent bleaching. Sections were photographed on Tri-X film with a Nikon UFX camera on a Zeiss Universal microscope equipped with UIRR epifluorescence optics.

Preparation of Antibodies to Cytotactin

Cytotactin was purified (20) by immunoadfinity chromatography using monoclonal antibody HNK-1 (an antibody with similar specificity to NC-1 [46]) immobilized on Sepharose CL-2B, followed by ion exchange chromatography on DEAE-cellulose. This material was used to immunize rabbits; the antibodies thus generated showed identical reactivity on Western blots and on frozen sections to the anti-cytotactin antibodies prepared and absorbed as previously described (20). With the exception of Fig. 8 H, which represents staining with monoclonal anti-cytotactin, rabbit antibodies against purified cytotactin were used in these studies.

Monoclonal antibodies were prepared against purified cytotactin and screened by ELISA (4) against the same material; clones that were positive by this analysis were then tested for their ability to immunoprecipitate radiolabeled cytotactin from the supernatants of cultured glial cells (20). In this study, the combined culture supernatants from four positive clones were used to confirm the immunohistochemical staining and immunoblotting observed with rabbit antibodies.

ImmunobLOTS

Tissues were extracted by homogenization using a Polytron homogenizer (Brinkmann Instruments Co., Westbury, NY) in 5 vol of 30 mM diethylyamine, 1 mM EDTA (pH 11.5), and shaken for 6 h at 4°C. The extracts were centrifuged at 10,000 g for 10 min, dialyzed against H2O, and 25-µl aliquots were subjected to gel electrophoresis (27). Immunoblots were done (45) using polyclonal antibodies to cytotactin as described above.

Results

Gastrulation

To assess the earliest appearance of cytotactin in the avian blastoderm, we examined sections from early embryos before and at various stages of gastrulation, with particular attention to areas of cell movements and epithelial–mesenchymal interconversions. Cytotactin first appears in the avian blastoderm at the time of gastrulation; it is not found in the blastoderm of fertile but incubated eggs. At the head process stage of gastrulation (stage 5), the molecule is present throughout the basement membrane (Fig. 1 A), separating the chordamesoderm (presumptive notochord) from the epithelium of the presumptive neural plate (i.e., at sites where gastrulation is complete). Staining is punctate at the level of the primitive streak where cells are ingressing to form the middle layer (Fig. 1 C). This pattern occurs reproducibly and may reflect the breakdown of the basement membrane in this region. Cytotactin is absent from Hensen's node (Fig. 1, E and F) and regions posterior to it (Fig. 1, G and H) in a later stage embryo (stage 9), which is already expressing cytotactin in developing anterior structures (see below). Thus, the appearance of cytotactin is subsequent to the regression of the primitive streak toward the posterior regions of the embryo.

Neurulation and Neural Crest Cell Formation

Because cytotactin was initially isolated as an NC-1 antigen, it was of particular interest to examine its distribution during the early formation of the neural tube and later during the formation and migration of neural crest cells. The anterior-posterior differential expression seen in the gastrulation stage embryo (Fig. 1) was repeated during neurulation (Fig. 2) and again during somite formation (Fig. 3, see below). In the stage 9 embryo, cytotactin is prominent in the basement membrane of the neural tube and notochord in the anterior (Fig. 2 A).
Figure I. Cytotactin expression during gastrulation. At the head process stage (stage 5), cytotactin is found in the basement membrane between the neural plate (np) and presumptive notochord (pn) in the anterior region of the blastoderm where gastrulation is complete (A). More posteriorly, it is absent from the basement membrane of the epiblast at the level of the primitive streak (ps) where extensive cell movement is still taking place (C). The primitive streak contains a small amount of cytotactin in a punctate distribution in the ingressing middle layer cells (C). B and D are phase images corresponding to A and C, respectively. In the posterior region of a later stage embryo (stage 9, 7 somites), cytotactin is absent both from Hensen’s node (hn) (E) and the primitive streak (ps) (G), where gastrulation is still occurring, although cytotactin staining has already become prominent in structures of the anterior regions (see Fig. 2). F and H are phase images that correspond to E and G, respectively. Bar, 50 μm.

and trunk (Fig. 2 B) regions; however, it is completely absent from the posterior regions (Fig. 2, C and D) even in regions where the notochord is well formed and neurulation has begun (Fig. 2 C). This pattern persists in the embryo through the third day of development (Fig. 2, E–H). At this stage, cytotactin is expressed only surrounding the neural tube, notochord, somites, and aorta (Fig. 2 E), a much more limited distribution pattern than fibronectin (Fig. 2 F). In the tail region (Fig. 2, G and H), no cytotactin is present (Fig. 2 G), although fibronectin staining (Fig. 2 H) clearly delineates the neural tube and notochord.

To examine further the appearance of cytotactin as somite
formation progressed, we prepared sagittal sections of 10-
somite embryos and again observed an anterior-posterior
differential expression in the somites of the posterior trunk
region (Fig. 3, A and B). Although staining was prominent in
the basement membranes of the most mature somites, two to
three of the newly formed somites and the pre-somitic seg-
mental plate contained no cytactin (Fig. 3, A and B),
indicating that its synthesis follows epithelialization of the
somite. This is in striking contrast to the expression of fibro-
nectin (Fig. 3 C) and laminin (Fig. 3 D) in the somites: fibro-
nectin strongly stains basement membranes of somites, seg-
mental plate, ectoderm, and endoderm, whereas laminin
strongly stains the basement membrane of the ectoderm but
stains somitic basement membrane at low levels in a punctate
pattern. Thus, it appears that during neurulation and somi-
togenesis, cytactin staining diminishes in the somatic ecto-
derm and increases in the basement membranes of the neural
ectoderm, notochord, and somites beginning anteriorly and
proceeding caudally. Again, in posterior trunk and tail re-

gions, formation of the neural plate, notochord, and somites
precedes the appearance of cytactin.

Cytactin is present during the formation of neural crest

**Figure 2. Cytactin staining at various axial levels of 2-d and 3-d embryos.**

(A–D) Transverse sections of a stage 9 embryo (7 somites) at the
level of the heart primordium (A), through the somites (B), and in regions posterior to the last formed somite (C and D) were stained with
affinity-purified rabbit anti-cytactin. (E–H) Transverse sections of a stage 18 embryo at levels posterior to the omphalomesenteric vein were
stained with affinity purified rabbit anti-cytactin (E and G) or rabbit anti-fibronectin (F and H). Note the restricted pattern of cytactin
staining (E and G) as compared with the extensive fibronectin staining in sister sections (F and H). Bar, 100 µm.
Figure 3. Comparison of cytotactin, fibronectin, and laminin distributions during somitogenesis. Parasagittal sections of a 10-somite embryo were stained with affinity-purified rabbit anti-cytotactin (A and B), rabbit anti-fibronectin (C), or affinity-purified anti-laminin (D). Arrows in A show area of enlargement in serial sections B, C, and D. A represents the entire embryo; three brain vesicles are clearly visible to the left. Five well-formed somites (s) plus the presomitic segmental plate (sp) are shown in B, C, and D. The last two somites show no cytotactin. Note also the low level of cytotactin staining in the ectoderm (e) compared with fibronectin (C) and laminin (D). Bar, 50 μm in A; 100 μm in B–D.

cells from the neural tube (Fig. 4, A–D). In the cephalic region of a 6-somite embryo (Fig. 4, A and B), staining is punctate in the presumptive neural crest area, similar to the distribution of cytotactin at gastrulation as middle layer cells delaminate from the epiblast (Fig. 1 C). At the 12-somite stage (Fig. 4, C and D), the mass of crest cells is completely surrounded by cytotactin before crest cell migration. At this stage, a comparison of cytotactin distribution (Fig. 4, E, G, and I) with that...
of fibronectin (Fig. 4, F, H, and J) shows that they appear together in the areas where crest cells will soon migrate, which includes the head region (Fig. 4, E and F), neural tube, notochord, and somites (Fig. 4, G--J). In contrast, cytotactin is not seen in regions where crest cells will not migrate, such as lateral plate mesoderm and ectoderm of the trunk (Fig. 4I), although in all of these areas fibronectin is prominent (Fig. 4J). The accumulated data indicate that cytotactin is clearly distinct in location from other ECM molecules such as laminin and fibronectin and that cytotactin is expressed in a pattern that is more specifically restricted to early neural crest pathways than either fibronectin or laminin.

The overall distribution pattern of cytotactin at different stages in the early embryo is schematically summarized in Fig. 5. At gastrulation, cytotactin appears throughout the basement membrane in the anterior regions of the embryo where gastrulation is complete (Fig. 5A). This differential anterior-posterior expression of the molecule is seen again during neurulation where formation of both the neural plate and the notochord preceeds cytotactin deposition in their basement membranes (Fig. 5B); this pattern repeats itself in the formation of somites (Fig. 5, B and C). In addition, cytotactin appears in the head mesenchyme, within the aorta, in the endocardium of the early heart, and in the early sclerotome. The molecule is not present in the myocardium or dermamyotome. The early distribution patterns foreshadow later appearances in the nervous system, developing cartilage, smooth muscle, and basement membranes of several tissues, and emphasize the restricted and sometimes transient distribution among certain tissues. The restricted tissue distribution of cytotactin, its order of appearance, and its prevalence in the nervous system suggest that it may function in a highly specialized fashion as compared with fibronectin or laminin.

**Cytotactin Expression in the Nervous System**

Cytotactin was initially isolated from embryonic brain tissue and was shown to be involved in glia–neuron interaction in in vitro assays (20). It was found to be expressed by glia but not by neurons of the central nervous system (CNS). It was therefore especially important to examine the appearance of this molecule in various regions of the nervous system. As discussed above, cytotactin is present in the basement membrane of the early CNS before its differentiation from an epithelial tube. By 6–7 embryonic days, cytotactin appears homogeneously, but at low levels in the spinal cord (Fig. 6A). During development, the level of cytotactin increases, and in the spinal cord does not appear to be preferentially expressed in particular areas such as white matter tracts that will become heavily myelinated (Fig. 6B). Similarly, cytotactin appears in the brain at 6–7 embryonic days (Fig. 6C) and is present in all areas of the brain during development. In the peripheral nervous system (PNS), cytotactin is associated with the early dorsal root ganglion as it forms between the neural tube and the dermamyotome (Fig. 6D). Staining is also prominent in cells within the more mature dorsal root ganglion (Fig. 6E).

In the developing sciatic nerve (Fig. 6F), cytotactin is associated with the perineurium of developing nerve, and with Schwann cells within the nerve. The molecule also appears in developing cartilage of both the axial (Fig. 6A) and appendicular skeleton (Fig. 6F); in more mature cartilage, it remains only in the perichondrium (Fig. 6B). Cytotactin is absent from the developing skeletal muscle and from the ectoderm of the limb bud (Fig. 6F) throughout its development.

During the development of the cerebellum, neurons of the external granule layer migrate to the internal granule layer using radial glial cells as guides (34). Consistent with its appearance on glial cells, but not neurons of the CNS, cytotactin is present in this radial pathway, from the early development of the cerebellum (not shown). As shown previously (20), cytotactin homogeneously stains the deeper cerebellar layers and remains associated with the radial glial fibers during migration of granule cells through the molecular layer.

The overall distribution pattern of cytotactin at different stages in the early embryo is schematically summarized in Fig. 5. At gastrulation, cytotactin appears throughout the basement membrane in the anterior regions of the embryo where gastrulation is complete (Fig. 5A). This differential anterior-posterior expression of the molecule is seen again during neurulation where formation of both the neural plate and the notochord preceeds cytotactin deposition in their basement membranes (Fig. 5B); this pattern repeats itself in the formation of somites (Fig. 5, B and C). In addition, cytotactin appears in the head mesenchyme, within the aorta, in the endocardium of the early heart, and in the early sclerotome. The molecule is not present in the myocardium or dermamyotome. The early distribution patterns foreshadow later appearances in the nervous system, developing cartilage, smooth muscle, and basement membranes of several tissues, and emphasize the restricted and sometimes transient distribution among certain tissues. The restricted tissue distribution of cytotactin, its order of appearance, and its prevalence in the nervous system suggest that it may function in a highly specialized fashion as compared with fibronectin or laminin.

**Cytotactin Expression in the Nervous System**

Cytotactin was initially isolated from embryonic brain tissue and was shown to be involved in glia–neuron interaction in in vitro assays (20). It was found to be expressed by glia but not by neurons of the central nervous system (CNS). It was therefore especially important to examine the appearance of this molecule in various regions of the nervous system. As discussed above, cytotactin is present in the basement membrane of the early CNS before its differentiation from an epithelial tube. By 6–7 embryonic days, cytotactin appears homogeneously, but at low levels in the spinal cord (Fig. 6A). During development, the level of cytotactin increases, and in the spinal cord does not appear to be preferentially expressed in particular areas such as white matter tracts that will become heavily myelinated (Fig. 6B). Similarly, cytotactin appears in the brain at 6–7 embryonic days (Fig. 6C) and is present in all areas of the brain during development. In the peripheral nervous system (PNS), cytotactin is associated with the early dorsal root ganglion as it forms between the neural tube and the dermamyotome (Fig. 6D). Staining is also prominent in cells within the more mature dorsal root ganglion (Fig. 6E).

In the developing sciatic nerve (Fig. 6F), cytotactin is associated with the perineurium of developing nerve, and with Schwann cells within the nerve. The molecule also appears in developing cartilage of both the axial (Fig. 6A) and appendicular skeleton (Fig. 6F); in more mature cartilage, it remains only in the perichondrium (Fig. 6B). Cytotactin is absent from the developing skeletal muscle and from the ectoderm of the limb bud (Fig. 6F) throughout its development.

During the development of the cerebellum, neurons of the external granule layer migrate to the internal granule layer using radial glial cells as guides (34). Consistent with its appearance on glial cells, but not neurons of the CNS, cytotactin is present in this radial pathway, from the early development of the cerebellum (not shown). As shown previously (20), cytotactin homogeneously stains the deeper cerebellar layers and remains associated with the radial glial fibers during migration of granule cells through the molecular layer.

To determine whether there was another example of cytotactin expression associated with cell movement in the CNS, we studied in detail the distribution of cytotactin during development of the retina. As the eye develops from the optic cup, cytotactin strongly stains the basement membrane surrounding both the lens and optic cup (presumptive retina) (Fig. 7D). By day 4, it is present at low levels on the optic fiber layer where axons are growing toward the optic nerve (Fig. 7E). As neurite migration begins within the retina, forming its complex laminar structure (E7), the molecule appears at low levels in a radial pattern through the retina and remains on the optic fiber layer (Fig. 7F). By day 9, cytotactin staining is observed in both the inner and the newly developing outer plexiform layer, and radially at low levels between these layers (Fig. 7G). By day 12, when ganglion cells have ceased their migration through the inner plexiform layer, radial staining is diminished while strong cytotactin staining remains in the inner and outer plexiform layers (Fig. 7H). This pattern is stabilized by day 17 (Fig. 7I), although staining of the outer plexiform layer is somewhat decreased. The data on cytotactin distribution in the retina and cerebellum clearly indicate the occurrence of cytotactin on glial cells at sites of known cell migration. This takes on particular significance in
light of observations that neuron–glia adhesion can be blocked by anti-cytotactin (20).

Appearance of Cytotactin at Nonneural Sites

We previously reported that cytotactin, although isolated from brain, was present at a number of nonneural sites in the embryo. We have now confirmed that this distribution is general for all smooth muscle, lung basement membrane throughout its development, and kidney basement membrane from mid-developmental through adult stages.

In the small intestine, cytotactin stains only the smooth muscle layer (Fig. 8A), while fibronectin is present at low levels in both the smooth muscle layer and the submucosa (Fig. 8B). Staining of the large ganglionic plexuses in the gizzard (Fig. 8, C and D) clearly indicates the presence of cytotactin (Fig. 8C) both in the smooth muscle and within some cells of the plexuses (a further indication of its association with peripheral glia), in contrast to fibronectin (Fig. 8D), which is present in the muscle but not within the nerve plexuses. Cytotactin is also associated with other types of smooth muscle. Arterial smooth muscles stain strongly (Fig. 8E), as shown above for the early development of the aorta. In the heart (Fig. 8F), valvular material, which is derived from the strongly cytotactin-positive endocardium (Fig. 2A and 4G), continues to express high levels of the molecule in striking contrast to the cardiac muscle which completely lacks the protein. As the erector muscles of the feathers develop, they express large amounts of cytotactin (Fig. 8G). High levels are also observed in bronchial smooth muscles (Fig. 8H). These results illustrate that cytotactin is associated both with smooth muscles and with peripheral glia of their associated nerves. Because smooth muscle cells are known to synthesize high amounts of cytotactin, these results suggest a functional role for the molecule in neuron-smooth muscle interaction.

Cytotactin is a prominent component of the basement membrane of both the developing lung and kidney but it appears in a different time sequence in each organ. In the lung, it is present when the initial lung buds form from the esophagus (20), and is prominent in the bronchi and parabronchi throughout their development (20; Fig. 8H). In the kidney, cytotactin is absent during the earliest stages of development, but appears surrounding the mesonephric tubules by embryonic day 7 (Fig. 8I). By day 13, it is strongly expressed as a component of both the tubular and glomerular basement membrane (Fig. 8H). These results indicate that cytotactin is an important component of basement membranes; while present together with other ECM molecules, it is expressed at different times and in different sequences.

Biochemical Properties of Cytotactin Expressed in Various Tissues

Cytotactin polypeptides of Mr 220,000 and a closely spaced doublet of Mr 200,000 and Mr 190,000 were previously isolated from buffer and Nonidet P-40 extracts of embryonic brain, and were found to be synthesized in culture by glial cells and by cells of 9-d embryonic gizzard, lung, and kidney (20). However, in preliminary experiments using other embryonic and adult tissues including adult brain, little cytotactin was extracted under these conditions. Efficient extraction of cytotactin from embryonic and adult tissues was obtained at high pH (30 mM diethylamine, 1 mM EDTA, pH 11.5). To determine the biochemical nature of the cytotactin antigens in various tissues at different ages, we did immunoblots of high pH extracts of whole embryos or isolated embryonic and adult organs (Fig. 9). A characteristic pattern of cytotactin polypeptides was observed in material extracted from E12 brain, kidney, lung, and gizzard (Fig. 9, lanes 1–4). No cytotactin was observed in tissues that exhibited no immunohistochemical staining, such as skeletal and heart muscle and liver (Fig. 9, lanes 5–7). Cytotactin was also extracted from 2-d whole embryos (Fig. 9, lane 8). The same polypeptides, including reproducible fragments of Mr 55,000 in brain and

Figure 5. Summary schematic diagram indicating restricted cytotactin appearance in the early embryo. Stippled areas represent the distribution of cytotactin in (A) a bead process stage embryo, (B) a 10-somite embryo (stage 10), and (C) a stage 18 (E3) embryo. Note the persistence of differential staining in anterior and posterior regions through the first 3 d of development.
Figure 6. Expression of cytotactin in the developing CNS and PNS. Transverse section through a 7-d embryonic trunk (A) shows low levels of cytotactin in the spinal cord, surrounding the notochord (n), and prominent staining in the developing cartilage (ct). By E13 (B) cytotactin has increased in the spinal cord and has disappeared from the maturing cartilage, remaining only in the surrounding perichondrium (pc). In the E7 optic lobe (C), cytotactin appears in all layers, most prominently on the developing stratum opticum (lower part of photograph). (D and E) The developing (E3) dorsal root ganglion (D, delineated by arrows) contains low levels of cytotactin that persist in the ganglion at day 13 (E). (F) The developing sciatic nerve (n) of an E4 embryo shows cytotactin within the nerve and in the surrounding perineurium, as well as in the developing cartilage (upper right). Cytotactin is absent from the limb ectoderm (e). Bar, 100 μm.

Figure 7. Differential cytotactin distribution in the retina. (A) In the E3 embryo, cytotactin is present in the basement membrane of the lens (l) and the presumptive retina (pr). (B) Cytotactin appears at E4 in the optic fiber layer (off) when axons are migrating along this layer toward the optic nerve. (C) As neurite migration begins within the retina itself (E7), cytotactin appears in a radial pattern through the retina while remaining on the off. (Intense staining at the bottom of the photograph is in cartilage surrounding the eye.) (D) By E9, cytotactin staining is observed in the inner (ipl) and at low levels on the outer (opl) plexiform layers, and the radial staining is diminished. (E) Radial staining disappears by E12, after migration is completed but remains on both the ipl and at low levels on the opl through E17. Bars, 50 μm.

Mr 65,000 in lung and gizzard, were recognized by monoclonal antibodies to cytotactin. These results indicate that cytotactin polypeptides are similar in all embryonic tissues and that the observed staining patterns reflect the expression of authentic cytotactin by these tissues in vivo.

In high pH extracts of adult tissues, cytotactin polypeptides of Mr 200,000 and Mr 220,000 were present in brain, kidney, and lung (Fig. 9, lanes 9–11). In the gizzard (Fig. 9, lane 12), the Mr 200,000 peptide was seen along with a major component of Mr 100,000. All of these forms of cytotactin were also recognized by a monoclonal antibody that recognizes an antigenic determinant retained in cytotactin molecules synthesized in the presence of tunicamycin (i.e., a non-carbohydrate determinant). Although we previously detected in adult...
Figure 8. Occurrence of cytotactin at extra-neural sites. (A and B) In the E12 small intestine, cytotactin (A) strongly stains the smooth muscle layer (sm) compared with fibronectin (B), which stains both smooth muscle and mucosal (m) layers at low levels. The presence of small amounts of cytotactin in the enteric ganglia is more apparent in the gizzard (C) where strong smooth muscle staining is also seen. Fibronectin (D) stains gizzard smooth muscle but not ganglia. (E) Transverse section through the anterior portion of the heart (E13) shows cytotactin staining in the arterial smooth muscles as they exit the heart. (F) Sagittal sections through the heart (E13) show cytotactin staining only within the endocardially derived valves but not in cardiac muscle. (G) Cytotactin strongly stains cells at the base of developing feather buds (E12), precursors to the erector smooth muscles of the mature feather. (H) Cytotactin is present in the E12 lung both in the basement membranes of the parabronchi and in the smooth muscles of the larger bronchi. (I and J) Cytotactin first appears in the kidney tubule basement membrane at E7 (I) and is expressed at high levels in both the tubule and glomerular basement membranes by E13 (J). Bar, 100 μm.
brain lower molecular weight components, these components are now minor or absent (Fig. 9, lane 9) using the high pH extraction conditions and the cytotactin antibodies used in this study. It appears that the M, 200,000 and 220,000 components are the major proteins recognized by both polyclonal and monoclonal anti-cytotactin antibodies in both embryonic and adult tissues.

**Cytotactin Distribution in the Adult**

To determine the relationship between cytotactin expression in embryonic and adult tissues, we examined its histological distribution in several regions in the adult. The distribution of adult cytotactin was found to be similar in most respects (Fig. 10) to cytotactin distribution in embryonic tissues. Cytotactin is found in the forebrain (Fig. 10A) and molecular layer of the cerebellum (Fig. 10B). Cytotactin is also expressed in the basement membrane of kidney and lung (Fig. 10, C and D), is prominent in both gut and arterial smooth muscles (Fig. 10, E and F), and is also seen in the smooth muscles of the feather (Fig. 10G). The adult skin provides one striking difference in cytotactin expression: cytotactin appears in the epidermal basement membrane of the adult skin in contrast to the embryonic skin in which no cytotactin is found.

**Discussion**

We have described here the distribution of a new cell-associated ECM molecule, cytotactin, during development of the chicken. The molecule is found during early embryogenesis in a more restricted pattern than that reported for ECM proteins such as fibronectin and laminin. It is present at a number of nonneural sites and, unlike laminin and fibronectin, appears during histogenesis of the nervous system in association with glia. Cytotactin is present in adult tissues in locations concordant with those in which it is found in embryonic tissues. The combined observations suggest that cytotactin appears in four major areas of critical morphogenetic and histogenetic activity in embryonic tissue: (a) at sites of major morphological changes during early embryogenesis, especially in association with cell movement; (b) in glial cells of the CNS and PNS; (c) in basement membranes of a variety of tissues in a temporal sequence distinct from other ECM proteins; and (d) in smooth muscles.

Immunofluorescent staining for cytotactin indicated that this cell-associated protein appears at the time of gastrulation in the basement membrane separating the epiblast from the underlying mesoderm. The molecule also appears at the level of the primitive streak in a punctate pattern that reflects the breakdown of the basement membrane as cells ingress to form the middle layer. At neurulation, cytotactin appears in the basement membrane of the neural tube, notochord, and somites in a striking pattern that appears earliest in the anterior, most mature portion of the embryo. Furthermore, this staining pattern delineates the early neural crest cell migratory pathways. When neural crest cells delaminate from the neural tube, the mass of cells is surrounded by cytotactin; their subsequent migratory pathways are lined by cytotactin. Although a similar distribution is observed for fibronectin, fibronectin also appears in widespread areas where crest cells do not migrate, such as the lateral plate mesoderm and lateral ectoderm, both of which lack cytotactin. These results raise the possibility that cytotactin, perhaps in conjunction with fibronectin (44), its receptor or other associated molecules such as the proteoglycan that co-purifies with cytotactin (20), directs or modulates cell migration in these areas. Moreover, cytotactin is found in several areas of active cell migration, both in the early embryo and in the CNS, including the sclerotomal cells of the dispersing somite, in the endocardium of the heart, in the cerebellum, and retina.

Cytotactin is synthesized by glia but not neurons of the CNS or PNS (unpublished observations). Immunolocalization data strongly support this observation and suggest that cytotactin may serve as an early marker of glial cell differentiation. Cytotactin is present in all regions of the brain and spinal cord, first appearing at approximately embryonic day 6 to 7. This timing and distribution pattern is consistent with times of large increases in the number of glial cells in the CNS (1). In addition, cytotactin staining occurs in a radial pattern in both the retina and cerebellum, where cell migrations occur, further correlating its appearance with sites of cell movement, and suggesting that the radial glia which provide a guide for neuronal migration in the cerebellum also express cytotactin. In the PNS, cytotactin both surrounds the peripheral nerves, as do laminin and fibronectin, and it is found within the nerves, suggesting its association with Schwann cells. Although both laminin and fibronectin strongly support neurite outgrowth, neither has been definitively found in the CNS. For this reason, cytotactin may have special significance in the CNS in vivo in supporting neuronal migration on glia. Moreover, a relationship between cytotactin expression and cell migration in both the CNS and PNS is likely to be particularly important. In a preliminary study, we have observed that both polyclonal and monoclonal antibodies to cytotactin inhibit cell migration in cerebellar slices in vitro (Chuong, C.-M., and G. M. Edelman, unpublished observations). A murine molecule called J1 glycoprotein having biochemical properties similar to cytotactin has recently been reported (26) to mediate neuron–astrocyte adhesion and to be expressed by astrocytes and oligodendrocytes but was
Figure 10. Distribution of cytotactin in the adult. Cytotactin appears in adult tissues in patterns similar to its distribution in embryonic tissues. Cytotactin is present in the adult forebrain (A), molecular layer of the cerebellum (B), kidney (C) and lung (D) basement membranes, and gizzard (E) and arterial (F) smooth muscle. In the adult skin (G–J), anti-cytotactin stains the erector smooth muscles (sm) and cells of the feather sheath (fs) below the rachis (r) or central part of the feather where the feather barbs (b) insert. In contract to the embryo, anti-cytotactin labels the basement membrane of the skin ectoderm (e) of the adult (I and J). H and J are the corresponding phase images of G and I, respectively. Bar, 100 μm.
Site-restricted Expression of Cytotactin

The expression of cytotactin in several nonneural areas, together with its known function in glia–neuron interactions, raises the possibility that, although it is an ECM molecule, cytotactin may nonetheless mediate cell–cell adhesion in certain situations. A particularly interesting case is that of smooth muscle cells. Thus, the cytotactin that remains after the disappearance of N-CAM, but that it is lost before differentiation into mature smooth muscle cells. Therefore, the cytotactin that remains after the disappearance of N-CAM may be involved both in adhesion between mature smooth muscle cells and in interactions of neurons with smooth muscle. The appearance of cytotactin in basement membranes of the kidney and lung may help to stabilize the tubular epithelia as do other ECM proteins, but because of the temporal differences in the appearance of cytotactin and other ECM molecules (especially in the kidney), it may also stabilize interactions among differing cell types necessary for tissue function.

The accumulated data allow us to construct a composite fate map as referred back onto the avian blastoderm (Fig. 11 A), providing a useful summary for comparing the different tissue expression of cytotactin (Fig. 11 C), the CAMs (Fig. 11 B), and fibronectin (Fig. 11 D). In the fate map of embryonic tissues, cytotactin expression is restricted to the neuroectoderm and only certain mesodermal and endodermal tissues, although the early ectoderm expresses cytotactin transiently. Thus, cytotactin, like the CAMs and fibronectin, is expressed by cells from all three germ layers but in a pattern restricted to only certain tissues and at certain times according to a schedule different from other ECM molecules such as fibronectin and laminin. The fate map of cytotactin distribution completely includes the map region of Ng-CAM and overlaps with many regions of N-CAM expression; in contrast, fibronectin and laminin are largely absent from CNS tissue. Although cytotactin is involved in glia–neuron interactions it does not appear to be the glial ligand for Ng-CAM (20). However, it remains possible that cytotactin may interact with N-CAM or Ng-CAM and in this way affect glia–neuron or other cellular interactions indirectly. Other potential cellular receptors for cytotactin in the brain and other tissues remain to be explored.

The expression of both CAMs and SAMs (such as cytotactin) in a spatially and temporally regulated fashion could help govern cellular interactions and movements during embryogenesis (11). While the CAMs are considered to be morphoregulatory proteins because they mediate cell adhesion and are expressed before morphological changes, cytotactin is expressed in areas of cell movement and subsequent to several major morphogenetic events such as neural tube formation and epithelialization of the somites. Thus, expression of cytotactin may stabilize previously formed structures as well as provide a new environment for cellular interactions or cell movement leading to further morphological processes. The distribution pattern of cytotactin indicates that map-restricted ECM molecules exist and raises the possibility that others may be found. Coordinated developmental regulation of gene expression of both CAMs and SAMs may be critical events leading to morphogenesis.

We thank Ms. Elizabeth McAnaney, Ms. Erica Weineger, and Ms. Jane Steinbeger for excellent technical assistance.

This work was supported by Public Health Service Grants AM-04256, HD-16550, HD-09635, NS-21629, and a Senator Jacob Javits Center of Excellence in Neuroscience Grant (NS-22789).

Received for publication 10 December 1985, and in revised form 24 January 1986.

References

1. Bensted, J. P. M., J. Dobbing, R. S. Morgan, R. T. W. Reid, and G. P. Wright. 1957. Neuroglial development and myelination in the spinal cord of the chick embryo. J. Embryol. Exp. Morphol. 5:428–437.

Crossin et al. Site-restricted Expression of Cytotactin

Figure 11. Fate maps comparing the distributions of CAMs, cytotactin, and fibronectin. (A) The presumptive areas of the blastoderm that will give rise to the different tissues has been established by various marking techniques (for review, see Vanke et al., [48]). (B) The distributions of the three known CAMs (N-CAM [], L-CAM [ ], and Ng-CAM [+]) are superimposed upon the classical fate map. Note that N-CAM- and L-CAM-containing tissues map to distinct and contiguous regions within the blastoderm and that Ng-CAM, a secondary CAM, appears later and only within neuroectodermal derivatives. (C and D) The distribution of tissues expressing cytotactin (C, [ ]) and fibronectin (D, [ ]) mapped onto the blastoderm. Compare the widespread occurrence of cellular fibronectin in most tissues, exclusive of the neuroectoderm, with the restricted distribution of cytotactin which is present in neuroectoderm and notochord, smooth muscle, urogenital system, in certain somite derivatives (sclerotome), and at low levels and at restricted times in ectoderm and endoderm. The vertical bar represents the primitive streak (PS); Ec, intraembryonic and extraembryonic ectoderm; En, endoderm; H, heart; LP, lateral plate (splanchno- and somatopleural mesoderm); N, nervous system; No, prechordal and chordamesoderm; S, somite.
2. Brackenbury, R., J.-P. Thiery, U. Rutishauser, and G. M. Edelman. 1977. Adhesion among neural cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. J. Biol. Chem. 252:6833–6840.

3. Carlson, B. M. 1981. Patten's Foundations of Embryology. McGraw Hill Book Co., Inc., New York. 672 pp.

4. Chuong, C.-M., and G. M. Edelman. 1984. Alterations in neural cell adhesion molecules during development of different regions of the nervous system. J. Neurosci. 4:2354–2368.

5. Covault, J., and J. R. Sanes. 1985. Neural cell adhesion molecule (N-CAM) accumulates in dennervated and paralyzed skeletal muscles. Proc. Natl. Acad. Sci. USA. 82:4544–4548.

6. Crossin, K. L., C.-M. Chuong, and G. M. Edelman. 1985. Expression sequences of cell adhesion molecules. Proc. Natl. Acad. Sci. USA. 82:6942–6946.

7. Damsky, C. H., J. Richa, D. Solter, K. Knudsen, and C. A. Buck. 1983. Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. Cell. 34:455–466.

8. Damloff, J. K., C.-M. Chuong, G. Levi, and G. M. Edelman. 1986. Differential distribution of cell adhesion molecules during histogenesis of the chick nervous system. J. Neurosci. In press.

9. Edelman, G. M. 1976. Surface modulation in cell recognition and cell growth. Science (Wash. DC). 192:218–226.

10. Edelman, G. M. 1983. Cell adhesion molecules. Science (Wash. DC). 219:450–457.

11. Edelman, G. M. 1984. Cell adhesion and morphogenesis: the regulator hypothesis. Proc. Natl. Acad. Sci. USA. 81:1480–1486.

12. Edelman, G. M. 1985. Cell adhesion and the molecular processes of morphogenesis. Annu. Rev. Biochem. 54:135–169.

13. Edelman, G. M. 1985. Expression of cell adhesion molecules during embryogenesis and regeneration. Exp. Cell Res. 161:1–16.

14. Edelman, G. M., and C.-M. Chuong. 1982. Embryonic to adult conversion of neural cell adhesion molecules in normal and staggerer mice. Proc. Natl. Acad. Sci. USA. 79:7036–7040.

15. Edelman, G. M., W. J. Gallin, A. Delouvée, B. A. Cunningham, and J.-P. Thiery. 1983. Early and developmental stages of two different cell adhesion molecules. Proc. Natl. Acad. Sci. USA. 80:4384–4388.

16. Gallin, W. J., G. M. Edelman, and B. A. Cunningham. 1983. Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells. Proc. Natl. Acad. Sci. USA. 80:1038–1042.

17. Grunet, M., and G. M. Edelman. 1984. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. J. Cell Biol. 98:1746–1756.

18. Hoffman, S., M. Hoffman, C.-M. Chuong, and G. M. Edelman. 1984. Polypeptide components and binding functions of neural-glia cell adhesion molecules. Proc. Natl. Acad. Sci. USA. 81:7989–7993.

19. Grunet, M., S. Hoffman, and G. M. Edelman. 1984. Two antigenically-related neural cell adhesion molecules of different specificities mediate neuron-neuron and neuron-glia adhesion. Proc. Natl. Acad. Sci. USA. 81:267–271.

20. Grunet, M., S. Hoffman, K. L. Crossin, and G. M. Edelman. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissue that mediates glia-neuron interaction. Proc. Natl. Acad. Sci. USA. 82:8075–8079.

21. Hamburger, V., and H. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49–92.

22. Hatta, K., T. S. Okada, and M. Takeichi. 1985. A monoclonal antibody disrupting calcium-dependent cell-cell adhesion of brain tissues: possible role of its target antigen in animal pattern formation. Proc. Natl. Acad. Sci. USA. 82:2789–2793.

23. Hoffman, S., B. C. Sorkin, P. C. White, R. Brackenbury, R. Mailhammer, U. Rutishauser, B. A. Cunningham, and G. M. Edelman. 1982. Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. J. Biol. Chem. 257:7720–7729.

24. Hoffman, S., and G. M. Edelman. 1983. Kinetics of homophilic binding by E and A forms of the neural cell adhesion molecule. Proc. Natl. Acad. Sci. USA. 80:5762–5766.

25. Hyafil, F., D. Morell, C. Babinet, and F. Jacob. 1980. A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. Cell. 21:927–934.