AN ULTRASTRUCTURAL EXAMINATION OF THE ROLE OF CELL MEMBRANE SURFACE COAT MATERIAL DURING NEURULATION

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ABSTRACT

Data from neural crest cultures indicate that cell surface coat material (CSM) is directly involved in cellular migration and events surrounding differentiation. To investigate whether the CSM also has a morphogenetic role, embryos of the amphibian *Ambystoma maculatum* were examined ultrastructurally throughout the stages of neurulation. Segments of the neural axis were fixed in glutaraldehyde-containing Alcian blue 8GX, which reportedly enhances preservation of CSM, and were postfixed in OsO₄ containing 1% lanthanum nitrate, which stains the CSM. The medial groove formed by the appearance of the neural ridges contains a large amount of CSM and numerous vesicles coated with lanthanum-positive material. In contrast, the lateral ridge surfaces are covered by a small amount of uniformly distributed CSM and a paucity of vesicles. As the ridges begin to fold there is a progressive increase in the amount of CSM within the presumptive neural tube region. Further convergence of the neural folds is accompanied by an increase of CSM at their leading edges. As the folds approximate each other, lanthanum-positive material physically bridges the gap. However, as the apposing tissue actually abuts to form the neural tube, no CSM is observed in the remaining interspace. The specific distribution and sequential accumulation of cell CSM during the events of neurulation strongly suggest its direct participation in the morphogenetic process.

The migratory and differentiative activities of cell populations during embryogenesis normally occur in a temporal and spatial pattern of awesome precision and complexity. Vogt's elegant fate-mapping experiments with amphibian embryos (30, 31) first demonstrated consistent directions of morphogenetic movement by various cell groups. Wilson's cellular reaggregation studies with sponges (37) introduced the concept of histogenetic specificity, i.e. a mixed-species population of cells will selectively reaggregate according to species. Galtsoff (10) extended this work by showing that mixed cells from various tissues of one species will reaggregate according to tissue.

The relevance of such data to developing general principles of morphogenesis generated experimental interest in the location and nature of a control mechanism associated with this predictable cellular movement and interaction. The accumulated facts from the classic studies of Holtfreter (17, 18)
and others (5, 7, 12–15, 21–24, 26, 29, 33–35) can be summarized in the statement that the structure and depositional pattern of extracellular materials, both at the cell surface and between cells, may impart an organizational specificity to tissue association and movement during the morphogenetic process.

It was the intent of this investigation to test the validity of this hypothesis in whole amphibian embryos undergoing neurulation. The advent of recent cytochemical (1, 20, 28) and biochemical (3, 6, 11) techniques has facilitated analysis of the morphology, distribution, composition, and molecular organization of these extracellular macromolecules. The data reported here suggest a correlation between the cell surface material (CSM) and the precision of organization during neurulation.

MATERIALS AND METHODS

Whole decapsulated embryos of the amphibian *Ambystoma maculatum* at developmental stages 14–24 (Harri-son) were fixed for 1 h in 2.5% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in Millonig’s buffer, pH 7.3. The fixative contained 0.5% Alcian blue 8GX (Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J.) which has been shown to enhance preservation of the cell surface coat material (1). The anterior trunk region of the neural axis was dissected free with iridectomy scissors, rinsed in buffer, and postfixed for 30 min in 1.0% osmium tetroxide (Engelhard Industries Div., Engelhard Minerals & Chemical Corp., Murray Hill, N. J.) containing 1.0% lanthanum nitrate (Fisher Scientific Co., Pittsburgh, Pa.) which stains the cell surface coat and renders it electron opaque. This reaction is facilitated by the previous fixation in an aldehyde-Alcian blue medium (28).

The tissue segments were dehydrated in a graded series of ethanol and propylene oxide solutions, infiltrated with 1:1 and 3:1 mixtures of Epon and propylene oxide, and embedded in Epon with 1.5% DMP-30 (Rohm and Haas Co., Philadelphia, Pa.) added as accelerator. Embedment was carried out in flat, rectangular molds using a dissecting microscope to insure consistent orientation of the neural axis. Polymerization occurred over 2 days at 37°, 45°, and 60°C. Sections were cut on a Porter-Blum MT2-B ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) with glass and diamond knives (E. I. DuPont de Nemours & Co., Wilmington, Del.), collected on copper grids, double-stained with uranyl acetate and lead citrate, and examined with a Philips EM300 electron microscope operating at 80 kV. Thick sections (1–2 μm) were also taken from each specimen and stained for light microscopy with 0.5% toluidine blue in phosphate buffer.

RESULTS

As the dorsal ectoderm of the late gastrula stages gives rise to the neural plate, a narrow layer of lanthanum-positive cell surface material is present above the plate. It spans the embryonic midline, conjoining the regions of the presumptive neural ridges, and is observable by both light (Fig. 1) and electron (Fig. 2) microscopy. A small amount of CSM is found on the cells lateral to this central formation. Its distance from the cell surface is limited, the bulk of the CSM apparently being contained by development of a dense line of aggregated material beyond which little CSM is observed (Figs. 1, 2).

All of these features persist and are amplified as the neural ridges develop. Lanthanum-positive material is preferentially associated with cell surfaces on the floor and sides of the neural groove and accumulates in the region above the midline (Fig. 3). The added thickness of this blanket of CSM is probably due to both its relative concentration as the neural ridges converge and its absolute increase in amount as its synthesis and release continue. The CSM becomes further organized in a precise linear array (Fig. 4) which at higher magnification appears thickly aggregated (Fig. 5).

The cell surface material appears as delicate fibrils radiating from a central structure (Fig. 6). The latter may be minute segments of cytoplasm, as it appears that at least some of the CSM is observed apart from the cell surface attached to what may be larger pieces of cytoplasm (Fig. 6), and many of the small clusters of CSM fibrils are attached to such amorphous material. Other fibrillar aggregates are associated with large vesicular structures present at the cell surface (Fig. 12, inset).

Development of the neural folds is accompanied by a relative upward displacement of the swath of CSM from the floor of the neural groove and the further concentration of the material into a thick dense bridge linking the folds (Fig. 7). Concomitant with the morphological changes which the cells at the base of the neural groove will undergo as the neural folds converge and the neural tube develops, accumulation of CSM ceases below the bridge and continues above it (Fig. 7), further thickening this structural link. Closer inspection of the bridge (Fig. 8) reveals a close packing of these CSM fronds, the original line of condensation...
being now so dense that it routinely fractures during sectioning (Fig. 7).

Apposition of the neural folds (Fig. 9) is associated with further concentration of the CSM. The discrete bridge fragments, losing attachment to the folds as they converge (Figs. 9, 10). The CSM present at the apex of the remaining neural groove and in the narrow cleft between the approximated

FIGURE 1 Light micrograph of the central portion of the neural plate (S-14). A line of lanthanum-stained cell surface material above the plate spans the embryonic midline and contacts the plate within this middle region. × 90.

FIGURE 2 Electron micrograph of the formation of cell surface material associated with the neural plate. A peripheral boundary line develops with most of the material contained beneath it. The cells at this stage are characterized by large round nuclei and dense yolk platelets occupying much of the cytoplasmic volume. × 560.
cell surfaces (Figs. 10, 11) progressively disappears. No CSM remains between the cells as contact actually occurs and the neural groove is obliterated.

DISCUSSION

As neurulation of the *Ambystoma* embryo proceeds, there is a discrete, temporal pattern of CSM deposition which appears to correlate functionally with the ongoing morphogenetic movements. Our data suggest that the bridge of CSM which appears at the neural plate stage linking the presumptive neural ridges serves to provide spatial orientation and guidance for the ridges and subsequent neural folds as they converge to form the neural tube. Upon apposition of the folds the CSM disappears from between adjoining cell membranes, an occurrence previously observed as plasma membranes fuse to form tight junctions (25). While the mechanism by which this structural link accompanies the convergence of the folds is unknown, it is noteworthy that it is consistently located in a region of cellular constriction. The possibility exists that the constrictive forces upon the neural epithelial cells may result in the mechanical extrusion of CSM which then forms the extracellular linear arrays necessary to link the folds.

A process of coordination of cellular and extracellular events in the successful accomplishment of neurulation would reasonably necessitate a reliable mechanism of release, extracellular organization, and destruction of cell surface material. It is widely accepted that CSM is synthesized within the Golgi apparatus and subsequently migrates to the plasma membrane (2, 3, 6). We have observed lanthanum-positive material in vesicles which appear intracytoplasmic (Fig. 12), although they may be connected to the surface in another plane of section, since we do not see them deeper in the cell interior and since they are apparently destined to fuse with the cell membrane and release their contents to the exterior (Fig. 12).

Of considerable interest are the vesicular structures seen external to the cell membrane (Fig. 12), which are of variable diameter and have a thick electron-dense shell of CSM (Fig. 12, inset). Their presence at the cell surface suggests a possible function in some aspect of CSM distribution (Fig. 12), but specifically how and why they are produced cannot be determined from this study. Their apparent importance is substantiated by a recent report (32) concerning alterations of surface morphology preceding palatal fusion in the mouse. The authors' scanning electron microscope observa-
FIGURE 4  Electron micrograph of an area comparable to that outlined in Fig. 3, demonstrating the remarkable linear organization of the CSM as it spans the underlying neural groove. × 880.

FIGURE 5  Higher magnification of Fig. 4 indicates that the bridge is composed of a thick aggregation of CSM with additional material beneath it. Bar = 1 μm × 10,180.

FIGURE 6  Electron micrograph of CSM as it appears adjacent to the fold surface (S) within the neural groove. Note the delicate fibrils emanating from a central structure and the heterogeneity of size and shape of CSM units. Their apparent ability to aggregate is well illustrated. Bar = 0.5 μm × 41,040.

tions include the presence of "filamentous material and small spherical bodies" along the areas of ultimate cellular contact. In view of the developmental similarities between fusion of palatal shelves and that of neural folds, it seems likely that these surface components would prove to be lanthanum-positive, and may also function in a similar fashion (8, 9, 16).
The movements of neurulation involve intracellular mechanisms which may in part be facilitated by, or even require supracellular coordination. Recent evidence on morphogenetic function during neurulation suggests that the driving force for the eruption and closure of the neural folds arises from a selective constriction and elongation of cells. The constrictive force appears to be related to the presence of microfilaments (4, 27), while cell elongation is associated with microtubules (4). It has been proposed that a collagenous basal lamina acts as a "foothold" to guide the elongating cells of the developing neural tube (15). The data reported above lead us to suggest that cell surface material is also contributory, by providing a structural link between the emerging folds assuring accurate.
**FIGURE 9** Light micrograph of the closure of the neural folds (S-19). The bridge of CSM becomes detached from its cellular connections (arrow) during the obliteration of the neural groove. × 28.

**FIGURE 10** Electron micrograph depicting the remnants of the CSM (R) within the remaining space between the neural folds (F). Some material aggregates along the cell surface and becomes wedged between the apposed folds (enclosed area). × 2,510.

**FIGURE 11** Higher magnification of the enclosed area of Fig. 10. Fold fusion is preceded by a gradual reduction in the opacity of CSM in the area where cell contact is imminent. Bar = 1 μm. × 27,930.
The mode of cellular release of CSM into the extracellular milieu is of considerable interest. In this regard CSM is observed to be attached to intact vesicles, certain of which are bound to the cell surface while others have no obvious cellular attachment. Small aggregates of CSM appear to be liberated from these heavily-stained structures (inset). Bar = 1 μm (inset, bar = 0.5 μm). x 25,650 (inset, x 51,300).

spatial alignment and fusion. In this regard it has been shown that the in vitro formation of nerve fiber membranous connections is preceded by contact with CSM (19), a relationship postulated much earlier by Weiss (33).

Thus the nature and the temporal pattern of secretion of carbohydrate-containing materials on to the cell surface may represent essential informational cues during the progressive determination of morphogenetic differentiation and in integrated aspects of multicellular function (26, 36).

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