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The Role of the Mesenchyme in Mouse Neural Fold Elevation I. Patterns of Mesenchymal Cell Distribution and Proliferation in Embryos Developing In Vitro

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ABSTRACT **Using the computer-assisted method of smoothed spatial averaging, spatial and temporal patterns of cell distribution and mitotic activity were analyzed in the cranial mesenchyme underlying the mesencephalic neural folds of mouse embryos maintained in roller tube culture. Total cell density increased in central and medial mesenchymal regions after 12 hr in culture, decreased after 18 hr, and showed a further decrease after 24 hr when the neural folds of the embryos had elevated, converged, and were fusing or fused. Mitotic activity, as measured by the ratio of ^{3}H-thymidine-labeled cells to unlabeled cells, was highest in the central mesenchyme at all culture times.**

Embryos were also cultured in the presence of diazo-oxo-norleucine (DON), which inhibits glycosaminoglycan and glycoprotein synthesis. After 24 hr in culture, neural folds of DON-treated embryos had failed to elevate. Total cell density increased in central and medial regions of the mesenchyme of DON-treated folds at 12 hr but showed no significant decrease in these regions with further culture. Mitotic activity was highest in the central mesenchyme of these treated embryos.

These results suggest that cell distribution patterns observed in the cranial mesenchyme during neural fold elevation in normal cultured embryos are not produced by regional differences in mitotic activity. Rather, we propose that cell distribution patterns in the central and medial regions of the mesenchyme result from expansion of a glycosaminoglycan-rich extracellular matrix that disperses cells from these regions and decreases their density. In DON-treated embryos, in which expansion of the mesenchyme is prohibited by the decreased glycosaminoglycan and glycoprotein content of the extracellular matrix, mitotic activity apparently determines these patterns.

INTRODUCTION

The cranial mesenchyme underlying the mouse mesencephalic neural folds is populated by mesenchymal cells embedded in a hyaluronate-rich extracellular matrix. In vivo elevation and convergence of mouse mesencephalic neural folds are correlated with an increase in cross-sectional area of the cranial mesenchyme, with a decrease in the density of cells residing in the central region of the mesenchyme and with a decrease in the concentration of hyaluronate (HA) in this region (Morris-Wiman and Brinkley, 1989). The decrease observed in mesenchymal cell density during elevation could result from a decrease in mitotic rate, from an increase in cell death, or from the expanion of the extracellular matrix of the cranial mesenchyme displacing cells from this region. The glycosaminoglycan hyaluronate is the major component of this extracellular matrix (Solursh and Morriss, 1977; Morriss and Solursh, 1978a,b; Heifetz et al., 1980). In biological and experimental systems, it is capable of a high degree of hydration with a concomitant increase in volume (Comper and Laurent, 1978). Previously we proposed a model for neural fold elevation and convergence in which the folds are "pushed" toward the dorsal midline by the directed expansion of an HA-rich extracellular matrix in the central regions of the cranial mesenchyme (Morris-Wiman and Brinkley, 1990). Such an expansion would be accompanied by a decrease in HA concentration in this region and the displacement of resident mesenchyme cells. However, a decreased synthesis or increased degradation of HA could also explain the observed decrease in HA content of the fold. This study was undertaken to determine if changes in patterns of mesenchymal cell distribution observed during neural fold elevation are the result of differential mitotic rates or of the expansion of the mesenchymal compartment. In a companion study, we have analyzed patterns of HA distribution and synthesis to determine the role of HA in mesenchymal expansion (Morris-Wiman and Brinkley, 1990).

If patterns of cell distribution within the cranial mesenchyme are determined by regional mitotic activity, one would expect that those regions of the mesenchyme displaying increased mitotic activity would become regions of increased cell density. The computer-assisted method of smoothed spatial averaging permits identification of areas within a histological section that have statistically significant differences in cell density and displays these differences as density contours (Connelly and Bookstein, 1983; Brinkley and Bookstein,

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1986). Using this method, we analyzed patterns of distribution of the total cell population within a cross section of the cranial mesenchyme underlying the mesencephalic neural folds. In order to detect regional differences in mitotic rates, we also analyzed distribution patterns of mesenchymal cells which had been labeled with ^{3}H-thymidine and the patterns of labeling ratios, i.e., the ratio of labeled to total cell densities. Our results indicate that patterns of total cell density do not reflect patterns of labelling ratios. Therefore, mitotic activity alone does not determine patterns of mesenchymal cell distribution.

If mesenchymal cell distribution patterns are effected by localized expansion of the extracellular matrix, then one would expect that, if such expansion were prohibited, mitotic activity would dictate which regions increase in cell density. To determine whether this is the case, embryos were cultured in the presence of diazo-oxo-norleucine (DON), a glutamine analogue that inhibits the formation of glucosamine, decreasing the glycosylation of glycoproteins and blocking glycosaminoglycan synthesis (Ghosh et al., 1960; Telser et al., 1965). Any mesenchymal expansion produced by an HA-rich extracellular matrix would then be prohibited by DON-treatment. Incubation of early somite embryos in the presence of DON has been shown to result in the failure of the cranial neural folds to elevate (Sadler et al., 1980; Morris-Wiman, 1988). The results of this study suggest that in DON-treated embryos the cranial mesenchyme does do not undergo the expansion characteristic of normal mesenchyme, and that patterns of cell distribution in treated mesenchyme are predicted by patterns of mitotic activity.

MATERIALS AND METHODS

Animals

Random-bred CD-1 mice were maintained in quarters with a dark cycle from 2000 to 0630 hr and fed Purina Mouse Chow and water ad libitum. Fertilization was assumed to occur between midnight and 0200 hr of the morning a vaginal plug was found. Timed pregnant females (day 0 = plug date) were sacrificed by cervical dislocation early on gestational day 8. Each uterine horn was removed to a petri dish containing Dulbecco's phosphate-buffered saline, in which the uterine wall was opened with watchmaker's forceps and the decidua removed. Two-somite embryos were dissected from the decidua with membranes intact and explanted to culture or immediately fixed.

Culture and Labeling

Embryos were explanted into a culture medium consisting of 1.0 ml, immediately centrifuged, heat inactivated rat serum (Steele and New, 1974), 0.5 ml FC-43 (O'Shea et al., 1982), 0.5 ml BGJ_b medium (Fitton-Jackson modification), 0.1 ml sterile water, and 0.05 ml garamycin (20 mg/100 ml). Diazo-oxo-norleucine (DON) (0.5 ml, 2.0 μg/ml, Sigma, St. Louis, MO) or sterile water was added to this medium before the addition of the embryos. Two embryos per bottle were gassed with a mixture of 5% O_2, 5% CO_2, and 90% N_2 (New et al., 1976) and rotated at 30 rpm at 37°C. The bottles were regassed at 12 hr or after the addition of ^{3}H-thymidine.

^{3}H-Thymidine (NEN, Boston, MA; sp. act. 79.8 Ci/mmol) was added to the culture medium in a final concentration of 2 μCi/ml at 0, 6, 12, and 18 hr of culture. Embryos were labeled for 6 hr and then removed from culture and fixed.

Histological Techniques

With the yolk sac and amnion removed, the embryos were fixed for 2 hr in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. They were then rinsed in buffer, dehydrated through graded alcohols, and embedded in glycol methacrylate (Sorvall). These embryos were then serially sectioned and 2-μm frontal sections were mounted on subbed slides. Sections from the mesencephalic regions of the cranial mesenchyme to be used for smoothed spatial averaging were Feulgen-stained and coated with Kodak NTB2 emulsion, diluted 1:1 with glass-distilled water. After a 2-week exposure, they were developed in D-19 for 4 min, rinsed in distilled water, fixed in Kodak Rapid Fixer, and allowed to air dry overnight. The slides were dipped in xylene before mounting in Permount. Slides for morphological evaluation and the calculation of the ratio of the nuclear-to-cell cross-sectional area were toluidine blue-stained, dipped in in xylene, and mounted in Permount.

Data Collection and Analysis

Two regions of the mesencephalic neural folds, anterior (just caudal to the diencephalon) and posterior (the most caudal section which did not include portions of the rhombencephalon), were studied from a minimum total of four embryos from at least three replicate cultures. No significant differences were found between anterior or posterior regions, right or left folds, for any of the measurements made. Therefore, the data reported represent the combined data from both regions and both right and left folds.

Using a Leitz Orthoplan microscope, photomicrographs were taken of complete frontal sections of each fold. A montage of photographic prints was produced at X618. From these photomontages, the cross-sectional area was computed from an outline of the mesenchymal perimeter of the fold using a Summagraphics digitizing tablet interfaced with a Tektronix 4054 computer running a simple measurement program.

Smoothed Spatial Averages

The computer-assisted method of smoothed spatial averaging has been described in detail elsewhere (Connelly and Bookstein, 1983; Brinkley and Bookstein, 1986) and is reviewed only briefly here. The positions of all labeled and unlabeled nuclei of mesenchymal and neural crest cells in the photomontages of each mesenchymal compartment were digitized together with a string of points representing the fold outline. A rectangular grid of smoothing boxes was placed over the image of each section, and the number of labeled and unlabeled cells was determined for each grid box. The number of labeled and unlabeled cells per 600 μm^2 and the ratio of labeled to unlabeled cells (the labeling ratio) were then computed for each grid box. These values were assigned to smoothing box centers. Contour maps representing statistically significant differences in these values were then drawn over the interior of the section. Contour maps were gray-level coded to aid in the discrimination of patterns. This method permits

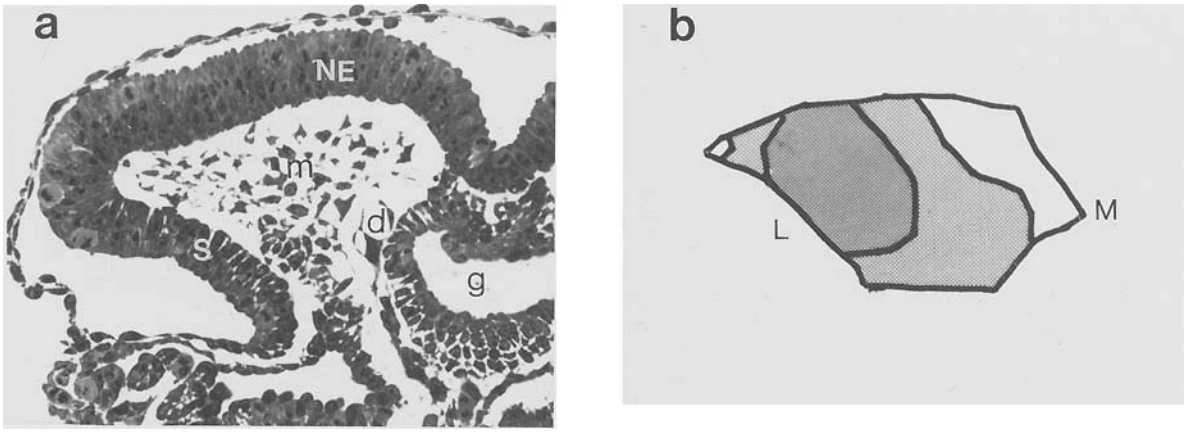

Fig. 1. **a:** Toluidine-blue-stained frontal section of the mesencephalic neural folds at time zero. The folds are small and biconvex. Both the neuroepithelium (NE) and the surface ectoderm (S) are columnar in organization. Cells are sparsely distributed throughout the cranial mesenchyme (m). d, Dorsal aorta; g, gut. ×205. **b:** Representative contour map of mesenchymal cell distribution at time zero. The contour map was generated using a smoothed spatial averaging algorithm (see methods) and then was gray-level coded as shown in the legend accompanying Figure 4 to aid in the discrimination of patterns. Cell density is observed to be increased in the lateral portions of the mesenchyme; the medial mesenchyme is relatively cell free. M, medial; L, lateral.

quantitative analysis of patterns of mesenchymal cell distribution within a particular neural fold. However, as yet no method exists for the statistical comparison of different plots. Therefore, the investigator must visually compare plots from different neural folds.

RESULTS

Morphological Changes

At time 0 the head folds of the presumptive mesencephalon are small and biconvex (Fig. 1a). The neural plate is composed of a columnar neuroepithelium (NE); it is bordered by a columnar surface ectoderm (SE). There is, as yet, no distinct demarcation between NE and SE. Fusiform mesenchymal cells appear evenly distributed throughout the cranial mesenchymal compartment, except in the region adjacent to the median portions of the neural groove, which remains cell free.

Controls

In controls after 6 hr of culture (Fig. 2), the head folds have increased significantly in size and are only slightly rounded. The NE is considerably thickened except in the median neural groove which is covered by columnar, wedge-shaped cells. There is a distinct demarcation between NE and SE at the dorsolateral edge of the neural fold. This is in part due to the change in the lateral NE from a columnar to a pseudostratified organization and in part due to a decrease in the height of the adjoining SE. The medial mesenchymal region adjacent to the median groove remains relatively cell free. The lateral regions of the mesenchyme now are populated by large, rounded, migrating neural crest cells.

The head folds after 12 hr of culture are no longer rounded, but are planar dorsally (Fig. 2). The neural folds are partially elevated, and the neural plate may appear V-shaped or have a slight mediad curvature. The NE had again increased in height, except in the shallow median neural groove, which is populated by wedge-shaped, columnar cells. The SE adjacent to the neuroepithelial–surface ectodermal (NE/SE) junction has decreased in height and has a stretched appearance that accentuates the boundary between NE and SE. The NE and SE of the neural fold intersect at a sharp angle, forming an elongated, elevated dorsolateral edge. The mesenchymal compartment has undergone some reorganization. Cells are distributed throughout the mesenchymal compartment, even into the formerly cell free area adjacent to the median neural groove. Lateral regions no longer are populated totally by migrating neural crest cells. Cells in the central and lateral regions of the mesenchyme have acquired a specific orientation in parallel with the transverse plane of the NE.

After 18 hr of culture, the dorsolateral edges of the neural folds are in the later stages of, or have completed, their convergence (Fig. 2). The median neural groove is shallow and detached from the notochord or foregut. The region subjacent to the median neural groove is now populated by mesenchymal cells. In this medial region, mesenchymal cells are widely separated and randomly oriented. No dead or pyknotic cells are observed. In more lateral aspects of the mesenchymal compartment, mesenchymal cells still retain their orientation.

The mesenchymal compartment has greatly expanded at 24 hr of culture, as indicated by the increased separation between the median neural groove and the gut or notochord. Cells inhabiting medial and central regions of the mesenchyme are widely separated and randomly oriented. Only in dorsolateral regions of the mesenchyme do cells still appear to be arranged in parallel to the base of the NE.

DON-treated embryos

After 6 hr of culture, the head folds of DON-treated embryos (Fig. 3) are, as yet, morphologically indistinguishable from those of control embryos. After 12 hr of culture, however, these folds are smaller than the controls and are rounded. The height of the NE is little

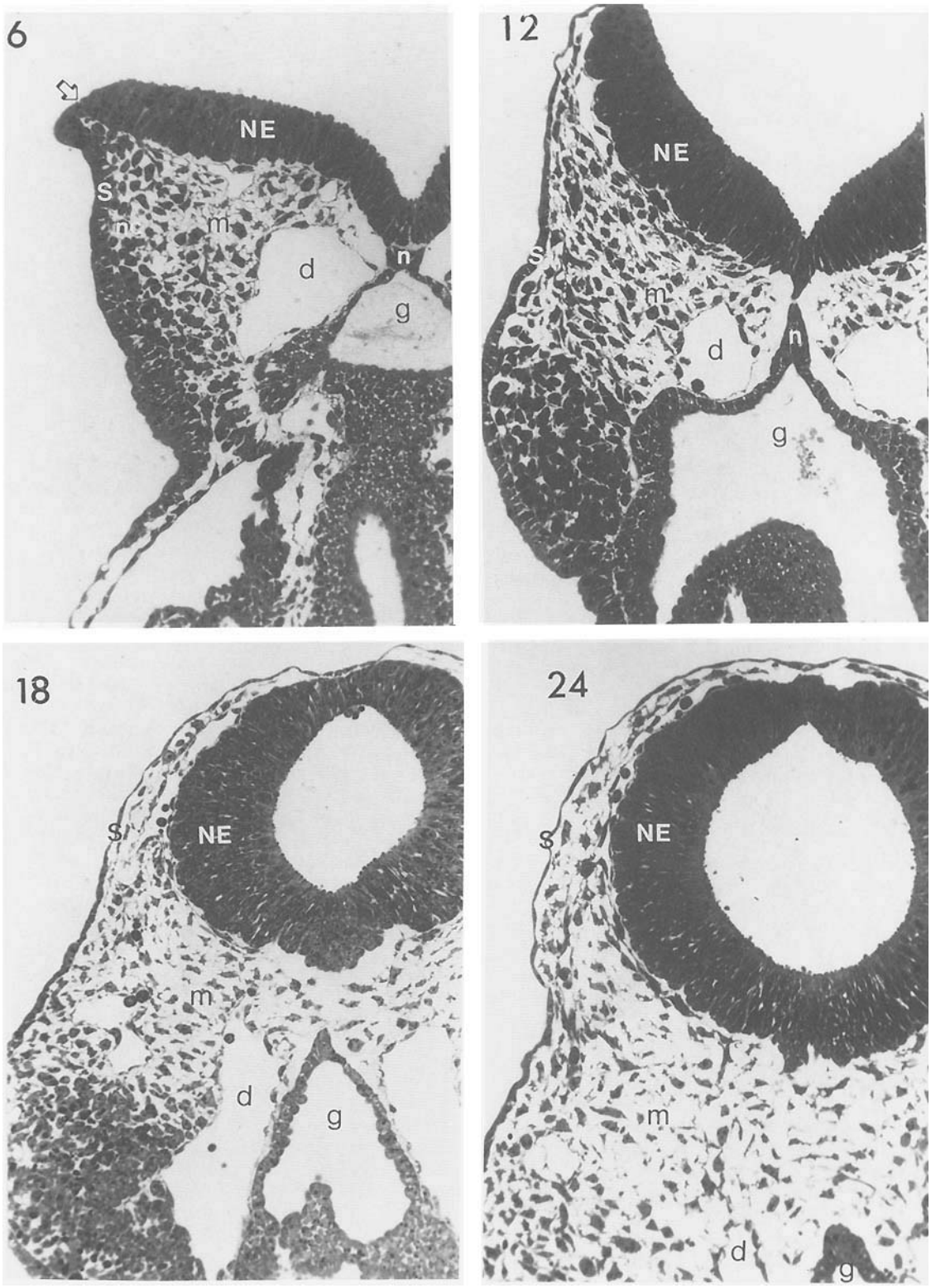

Fig. 2. Toluidine-blue-stained frontal sections of the mesencephalic neural folds of control embryos after 6, 12, 18, and 24 hr of culture. Elevation of the neural folds is accompanied by a thickening of the neuroepithelium (NE) and a thinning of the adjacent surface ectoderm (S). Mesenchymal (m) cell density increases at 6 and 12 hr of culture. At 12 hr, mesenchymal cells appear to be preferentially aligned in parallel to the transverse plane of the neuroepithelium. At 18 and 24 hr, mesenchymal cell density is decreased and orientation appears more random. The considerable expansion of the cranial mesenchyme at 18 and 24 hr is accompanied by the separation of the median neural groove and the gut (g). d, Dorsal aorta; n, notochord; nc, neural crest; arrow, NE/S junction. ×205.

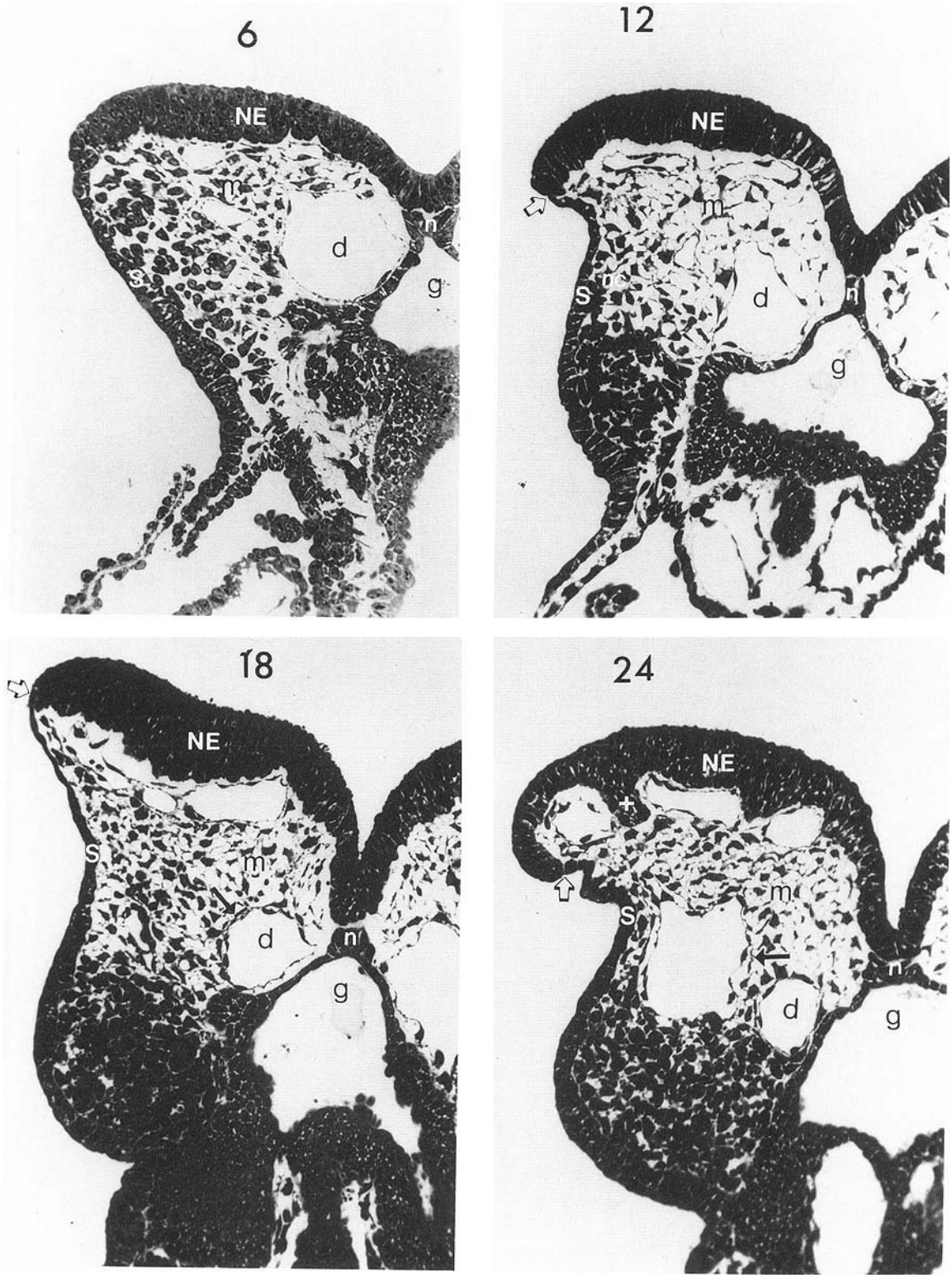

Fig 3. Toluidine-blue-stained frontal sections of the mesencephalic neural folds after 6, 12, 18, and 24 hr of culture in the presence of DON. The neuroepithelium (NE) of DON-treated folds appears to thicken slightly with culture but not nearly to the extent observed in control folds (cf. Fg. 2). The basal surface of the neuroepithelium may be scalloped and indent into the mesenchymal compartment. The median neural groove is increased in depth and remains attached to the notochord (n) or gut (g) even after 24 hr of culture. The cranial mesenchyme (m) of treated folds does not undergo the expansion characteristic of control folds at 18 and 24 hr of culture. Much of the mesenchymal compartment is occupied by the greatly dilated blood vessels. Mesenchymal cells are often aligned in parallel to the endothelium of these vessels (arrow at 24 hr). The vessels underlying the neuroepithelium are separated from the neuroepithelial basement membrane by a definite cell-free space. S, Surface ectoderm; d, dorsal aorta; nc, neural crest; open arrow, NE/S junction. ×205.

Table 1. Changes in mesenchymal cross-sectional area, mesenchymal cell density and labeling ratio during fold elevation and convergence[1]

Culture time (hr)	N[2]	AREA (10^3 μm^2)	No. of cells per fold	Cell density (cells per 600 μm^2)	Labeling ratio
Controls					
0	6	15.08 ± 3.66	48.00 ± 10.48	1.75 ± 0.44	—
6	5	27.78 ± 3.72	107.96 ± 23.74*	2.34 ± 0.48*	0.47 ± 0.08
12	5	35.59 ± 7.02*	138.25 ± 34.29*	2.36 ± 0.21	0.35 ± 0.09*
18	6	46.93 ± 8.10*	158.46 ± 24.95*	2.13 ± 0.34	0.57 ± 0.10*
24	6	68.00 ± 13.94*	218.36 ± 33.18*	1.96 ± 0.33	0.65 ± 0.08*
DON-treated					
6	6	26.39 ± 4.69*†	89.71 ± 19.27*†	2.00 ± 0.39	0.53 ± 0.12
12	4	30.70 ± 2.37	92.88 ± 8.62†	1.84 ± 0.29	0.18 ± 0.15*†
18	9	34.15 ± 9.43† (27.63 ± 4.79)[3,†]	121.66 ± 21.95*,†	2.25 ± 0.65* (2.74 ± 0.67)[3]	0.34 ± 0.08*†
24	6	41.67 ± 6.98* (29.88 ± 8.65)[3,†]	103.75 ± 27.39*†	1.50 ± 0.56*† (2.28 ± 0.95)[3]	0.36 ± 0.09*

[1]Values are mean ±standard deviation.
[2]N indicates the number of embryos examined.
[3]Values in parentheses or corrected for blood vessel area (see text).
*Mean is significantly different ($P \leq 0.01$) from that of preceding stage by analysis of variance.
†Indicates significant difference ($P \leq 0.01$) between DON-treated and control of the same culture age.

changed from that at 6 hr. Laterally the NE may have thickened slightly and is pseudostratified. The median neural groove has increased in depth and is covered by columnar, wedge-shaped NE cells. The basal surface of the NE may be scalloped. A distinct NE/SE junction owes its existence to the slight thinning of the adjacent SE. However, the lateral edge of the neural fold formed by this NE/SE interface is bent ventad. Cells are few in number and are dispersed throughout the mesenchyme. They appear randomly oriented. The formerly cell-free medial region of the mesenchyme adjacent to the median neural groove now harbors mesenchymal cells. Lateral regions of the mesenchyme possess a few migrating neural crest cells. In the central and medial regions of the mesenchyme, a prominent cell-free space separates the neuroepithelial basement membrane (NE/Bm) from subjacent blood vessels.

At 18 hr, the head folds are slightly rounded (Fig. 3). A shallow midfold ventral flexure is apparent in the neural plate. The median neural groove is deep and lined with a columnar NE. The NE comprising the ventral flexure and in the central fold is thickened and pseudostratified, but the NE thins toward its lateral edge. The distinct NE/SE interface is bounded by a thinned, squamous SE. The lateral edge of the neural fold at the NE/SE junction remains turned slightly ventrally as at 12 hr. A cell-free space still intervenes between the NE/Bm and subjacent blood vessels. These blood vessels are enlarged and may be interconnected to form a single large vessel, which separates the NE of the central fold entirely from the rest of the underlying mesenchyme. Mesenchymal cells are often aligned with their long axes parallel to the endothelium of the vessels, giving these vessels the appearance of having more than one endothelial coat.

After 24 hr of culture, the head folds are rounded and appear translucent macroscopically due to the expansion and engorgement of the vessels of the cranial mesenchyme (Fig. 3). Much of the space in the mesenchymal compartments is occupied by enlarged blood vessels subjacent to the NE, sometimes interconnecting with the cardinal vein and dorsal aorta. Mesenchymal cells are crowded into small bands between these vessels and, as at 18 hr, are often aligned parallel to the endothelial boundaries of vessels to form a mesenchymal border 1 or 2 cells thick. The NE appears to have decreased in height, particularly in the median neural groove and at the dorsolateral edge of the fold. The somewhat splayed median neural groove is covered by a columnar NE. Laterally the NE has also become columnar and bends ventrally over the fold's dorsolateral edge to meet the adjacent SE. The SE is no longer squamous, but is cuboidal and may be buckled. The NE of the central fold is often slightly disorganized. Its basal surface may be scalloped or appear to invade the subjacent mesenchyme between blood vessels.

Changes in Mesenchymal Cell Density

The mesenchymal cell density, expressed as the number of cells per 600 μm^2, was computed for each fold. Also measured were the cross-sectional area of the cranial mesenchyme and the total number of cells in the mesenchyme (Table 1).

Controls

The total number of cells in the cranial mesenchyme increases significantly with culture time, with the greatest increase occurring between 18 and 24 hr of culture. Likewise, the area of the mesenchyme increases significantly with culture time; large increases accompany transitions from 12 hr to 18 hr and from 18 hr to 24 hr in culture. The twofold increase in cell number from 0 to 6 hr in culture results in a significant increase in the cell density in the mesenchyme. The decrease in cell density at 18 and 24 hr of culture is concomitant with the large increases in mesenchymal cross-sectional area at these time periods.

DON-treated

Although the total number of cells and the cross-sectional area of the cranial mesenchyme of DON-treated embryos increase with time in culture, the increases are smaller than those observed in control mesenchyme. There is a significant increase in cell number after 6 hr of culture; however, the total number of cells in the mesenchyme is significantly less than in the controls. The area of DON-treated mesenchyme does not differ significantly from that of controls at 6 hr. Therefore, the cell density at 6 hr is lower in DON-treated mesenchyme than in controls. There is no significant increase in total cell number or in mesenchymal cross-sectional area in DON-treated cranial mesenchyme at 12 hr of culture over the values at 6 hr in DON-treated embryos. Further culture results in a significant increase in total cell number but no increase in mesenchymal cross-sectional area. Thus, the cell density of the fold at 18 hr is significantly increased over that at 12 hr.

At 24 hr the total cell number and the cell density are significantly lower than at 18 hr in DON-treated embryos. The mesenchymal cross-sectional area is significantly increased. However, this increase in area appears to be the result of the enlargement of the cross-sectional area of the cranial blood vessels. If the cross-sectional area of these vessels is subtracted from the total mesenchymal cross-sectional area at 18 and 24 hr of culture, the resultant corrected mesenchymal cross-sectional areas do not differ significantly from one another. However, the corrected cell density for DON-treated mesenchyme at 24 hr is still significantly decreased from the corrected cell density at 18 hr. This decrease in cell density is, therefore, not the result of an increase in mesenchymal area in the transverse plane. Alternatively, the decrease could be the result of an increased mesenchymal cell death; however, very few necrotic cells are observed in the mesenchyme at 24 hr of culture. It is more likely that the decrease in mesenchymal cell density at 24 hr is the result of decreased mesenchymal cell numbers in the transverse plane produced by the rostrocaudal expansion of the mesenchyme with the engorgement of the cranial vasculature.

Patterns of Mesenchymal Cell Distribution

Controls

At time 0, cells are more concentrated in the central and lateral regions of the cranial mesenchyme (Fig. 1b). The mesenchyme adjacent to the median neural groove is relatively cell free. After 6 hr of culture, mesenchymal cells remain concentrated in the lateral mesenchyme, particularly in the more dorsal and ventral regions (Fig. 4). An increase in cell density is apparent in the central core of the mesenchyme at this time, and the increase extends into the dorsomedial mesenchyme subjacent to the rim of the median neural groove. The region adjacent to the median neural groove and the dorsal aorta remains almost cell free. Analysis of patterns of labeled cell distributions shows that areas of high density of labeled cells coincide with areas of high total cell density. Labeling ratios, however, are elevated within the central core of the mesenchyme, decreasing laterally and medially.

Patterns of cell distribution after 12 hr of culture (Fig. 4) do not differ radically from those at 6 hr. The most lateral portions of the mesenchyme are areas of higher cell densities than the more medial regions. The region of highest mesenchymal cell density corresponds to the ventrolateral fold which is heavily populated by neural crest cells. The dorsolateral mesenchyme at the base of the elevating mesenchyme edge and the dorsocentral fold are also heavily populated. The mesenchyme enclosed by the NE and SE of the elevating fold edge is sparsely inhabited and the medial mesenchyme remains relatively cell free. Patterns of labeled cell density are similar to those of total cell density. The highest labeling ratio is observed in the central core of the mesenchyme as at 6 hr of culture. However, an increase in the labeling ratio is also apparent medially.

After 18 hr of culture, the mesenchyme is characterized by having only two regions of high cell density. One lies at the base of the elevating dorsolateral edge of the neural fold; the other is in the ventrolateral portion of the mesenchyme, which contains a portion of the first arch. The central mesenchyme has decreased in cell density. The distribution of labeled cells follows the same pattern as the total cell density. Labeling ratios are elevated on a diagonal from the dorsomedial to the ventralateral mesenchyme through the central mesenchymal core. An area of increased mitotic activity also exists in the mesenchyme at the base of the dorsolateral fold edge.

After 24 hr of culture, the cell density is low in most of the mesenchyme (Fig. 4). Only the dorsolateral and the ventrolateral mesenchyme that were heavily populated at 18 hr show high total cell and labeled cell densities. Labeling ratios are increased in all dorsal portions of the mesenchyme, but are particularly high in the dorsomedial and dorsocentral regions.

DON-treated

After 6 hr of culture the patterns of total and labeled mesenchymal cell distribution in DON-treated embryos (Fig. 5) are indistinguishable from those of controls. In some cases, however, labeling ratio patterns differ; in DON-treated mesenchyme, lateral regions of the mesenchyme often have increased labeling ratios. After 12 hr in culture, although the total cell density of the DON-treated mesenchyme is considerably lower than that of controls, the patterns are not radically different from those observed in control mesenchyme. As in controls, the highest cell densities are in the more lateral mesenchymal regions, extending into central regions. Medial regions are relatively cell free. Unlike controls, however, DON-treated mesenchyme is very sparsely populated in its more dorsal regions. Labeled cells are found in highest concentration in the ventrolateral region of treated mesenchyme and in a small region of the mesenchyme core compressed into a diagonal band extending from ventrolateral to dorsolateral regions. Labeling ratios are elevated in the dorsomedial and medial portions of the mesenchyme. The number of labeled cells, however, is considerably less than at 6 hr; and the resulting labeling ratio is significantly decreased (Table 1).

Areas of high total cell density are most prominent in the lateral portions of DON-treated mesen-

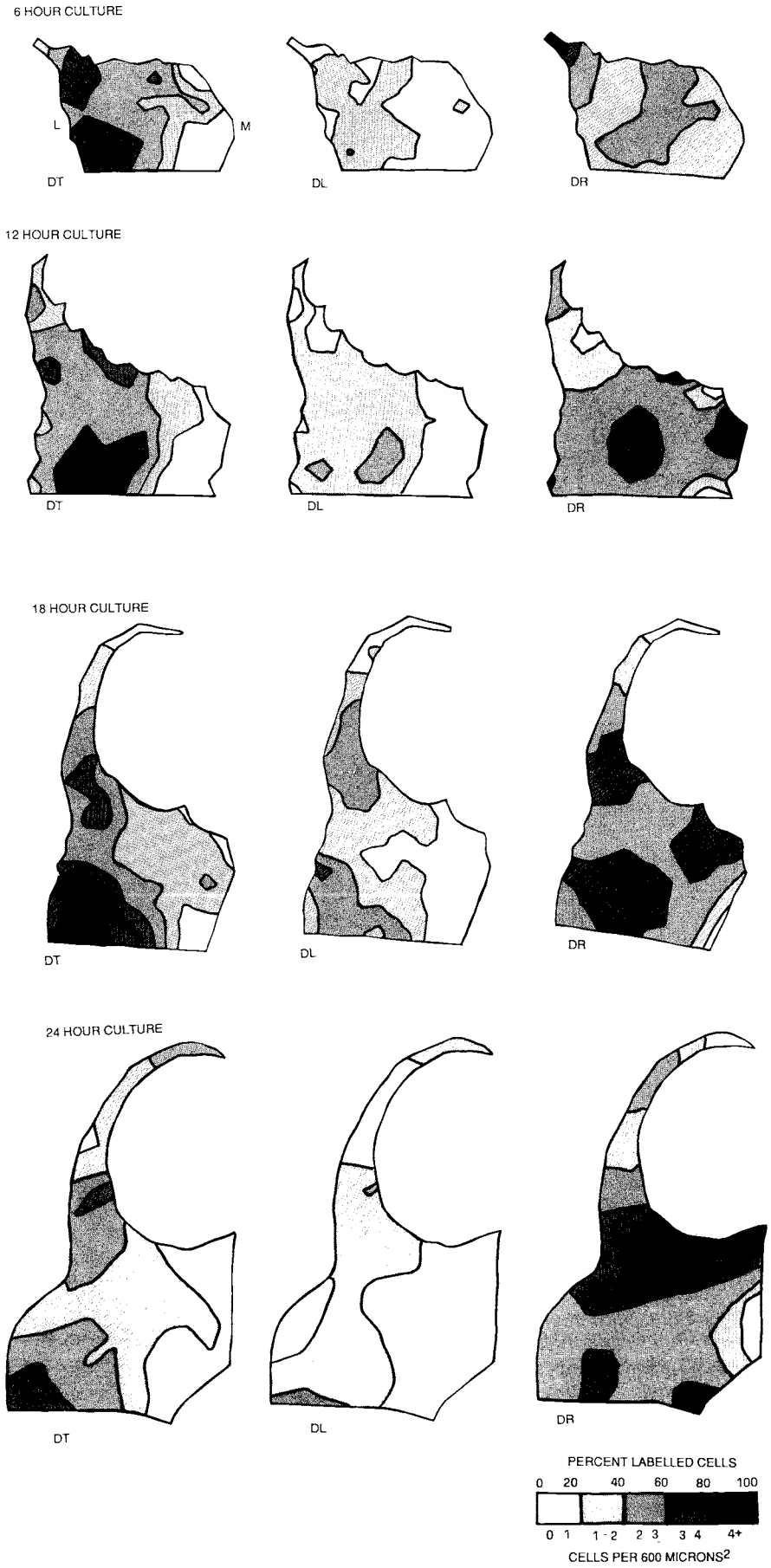
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Fig. 4.

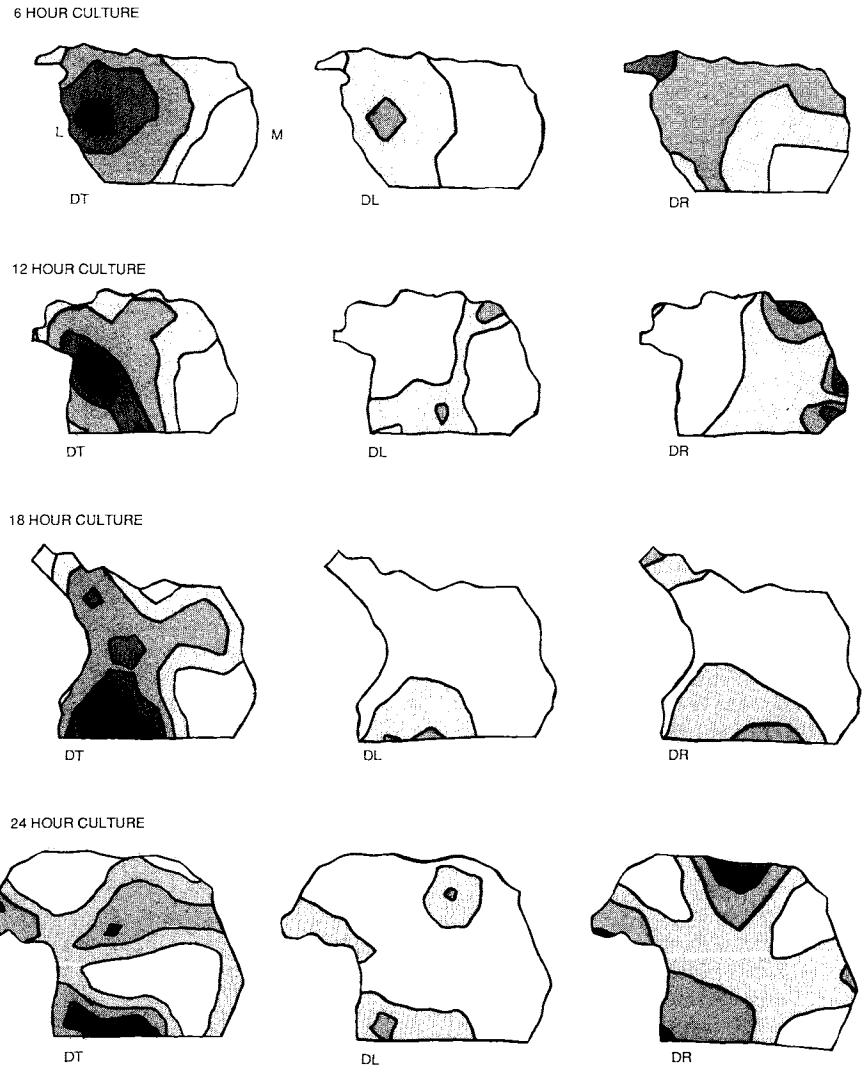

Fig. 5. DON-treated embryos. Representative contour maps of total mesenchymal cell density (DT), labeled cell density (DL), and labeling ratio (DR) of the cranial mesenchyme of DON-treated neural folds after 6, 12, 18, and 24 hr of culture. Maps were coded as in Figure 4. In treatedfolds, total mesenchymal cell density is observed to increase in the central mesenchyme. Areas of increased labeled cell density correspond to areas of increased total cell density. Labeling ratios are greatest centrally and medially. L, lateral; M, medial.

chyme after 18 hr of culture (Fig. 5), but they also extend through central regions into the dorsomedial mesenchyme. The only area of high labeled cell density is in the ventrolateral mesenchyme, which includes a portion of the first arch; the labeling ratio is also high in this region. The mean labeling ratio is increased over that observed at 12 hr, but is still significantly less than that of controls at 18 hr (Table 1).

After 24 hr of culture, mesenchymal cells are compressed into a small band in the mesenchymal core by the expansion of the cranial vasculature. The ventrolateral mesenchyme remains the region of greatest cell density, as at 18 hr. A pocket of increased mesenchymal cell density is also observed in the compartment enclosed by the NE/SE junction. Regions of higher labeled cell density correspond to the ventrolateral and dorsolateral mesenchyme. These two regions, along with the dorsomedial mesenchyme, possess elevated labeling ratios. The labeling ratio of the DON-treated mesenchyme is significantly lower than that observed in controls (Table 1).

Fig. 4. Control embryos. Representative contour maps of total mesenchymal cell density (DT) labeled cell density (DL), and the ratio of labeled to total cell density (labeling ratio, DR) of the cranial mesenchyme of the mesencephalic neural folds after 6, 12, 18, and 24 hr of culture. These maps are gray-level coded, as shown in the legend, to permit pattern discrimination. The total cell density is observed to decrease with time in culture, particularly in the central and medial mesenchyme regions. Labeled cell density patterns are similar to those of total cell density. However, the labeling ratios are increased in the central mesenchymal core at all culture times. L, lateral; M, medial.

DISCUSSION

Using the computer-assisted method of smoothed spatial averaging, we have analyzed patterns of cell distribution, as well as mitotic activity, in the cranial mesenchyme during the in vivo elevation of the mouse mesencephalic neural folds. Our results suggest that, in normal fold morphogenesis, patterns of mesenchymal cell distribution are not determined by a differential mitotic rate. The more lateral regions of the cranial mesenchyme were more densely populated at all stages of neural fold elevation. However, these regions often possessed lower labeling ratios than did the central and medial mesenchyme regions, indicating a decreased mitotic activity as compared with these other regions. The lateral mesenchyme contains the migration pathway for neural crest cells (Nichols, 1981), and the high cell density observed in this region is mainly due to this transient population. Mesenchymal cell density decreased in the central and medial mesenchyme as elevation and convergence proceded. Yet these regions were associated with high labeling ratios. Therefore, in the cranial mesenchyme of the mesencephalic neural folds of cultured mouse embryos, patterns of labeling ratios, or mitotic activity, do not predict patterns of mesenchymal cell distribution. If mitotic activity alone determined cell distribution, one would expect to see an increased cell density in the medial and central regions of the cranial mesenchyme as the folds elevated and converged.

The mesenchymal cell distribution patterns observed could be the result of an increase in cell death or at the displacement of cells from the central and medial regions of the mesenchyme. No appreciable cell death was observed in any region of the normal mesenchyme. The displacement of cells from the central and medial mesenchyme could be by active migration or by passive dispersal with the expansion of the mesenchyme. Passive cell translocation by extracellular matrix expansion has been reported to occur in the transition of epithelium to mesenchyme in the sclerotome (Solursh et al., 1979), in the growing limb bud (Sugrue, 1979), and in the remodeling of the secondary palate (Brinkley and Bookstein, 1986). Circumstantial evidence suggests that in the cranial mesenchyme of the neural folds, cell-distribution patterns are the result of matrix expansion. The decrease in cell density in the medial and central regions of the mesenchyme is concomitant with a significant expansion of the mesenchyme as indicated by an increase in mesenchymal cross-sectional area (Table 1). In a companion study, we have demonstrated that the in vitro expansion of the mesenchyme is accompanied by a decrease in hyaluronate (HA) concentration in central regions of the cranial mesenchyme (Morris-Wiman and Brinkley, 1990). HA is a highly charged molecule that may intermesh with itself or other extracellular matrix components to form large networks that sequester water. This property of HA is the probable basis for the capacity of HA-rich matrices in the sclerotome, limb buds, and palate for sudden expansion with concomitant cell displacement (Solursh et al., 1979; Sugrue, 1979; Brinkley and Morris-Wiman, 1987). The expansion of the HA-rich extracellular matrix of the central mesenchyme would lead to displacement of the resident mesenchymal cells, decreasing cell density.

If the observed decrease in cell density in the central and medial mesenchymal compartments results from passive cell displacement with the expansion of an HA-rich extracellular matrix, disruption of HA synthesis would interfere with the normal patterning of mesenchymal cells. DON interferes with the synthesis of glycosaminoglycans and glycoproteins by inhibiting the glutamine-dependent formation of glucosamine from glucose 6-phosphate (Ghosh et al., 1960; Telser et al., 1965). DON also binds irreversibly to an enzyme responsible for purine synthesis (Levenberg et al., 1957; Hartman and Buchanan, 1959; Buchanan, 1973). The effects of DON on glucosamine synthesis can be overridden by the addition of excess glutamine (Greene and Kochhar, 1975; Greene and Pratt, 1977; Ekblom et al., 1979; Humerinta et al., 1979; Lebedeva et al., 1986). The effects of DON on purine synthesis are not overcome by glutamine addition but require the addition of purine analogs or precursors (Chu and Henderson, 1972; Hartman and Buchanan, 1959). We have observed that neural folds cultured in the presence of DON and excess glutamine (2 mM) proceded normally through neural fold elevation (Morris-Wiman, 1988). However, the neural folds of embryos cultured in DON and the purine analog amino imidazol carboxamidine (AIC) did not elevate after 24 hr of culture. The inability of AIC to restore normal fold morphogenesis suggests that the effects of DON on neural-fold morphogenesis are the result of its interference with normal glucosamine synthesis that could be alleviated by the addition of excess glutamine. We observed, however, that the NE and SE layers of embryos cultured in DON with AIC appeared unaffected histologically, unlike those incubated in DON alone, suggesting that the effects of DON incubation may not be restricted to inhibition of glucosamine synthesis.

Analysis of patterns of cell distribution and labeling ratios in the cranial mesenchyme of DON-treated embryos revealed that, despite lower labeling ratios in the treated mesenchyme, the patterns of labeling ratios were similar to those of controls. In both, the central and medial mesenchymal regions possessed the highest values. Unlike in controls, however, in DON-treated mesenchyme these regions became regions of increased cell density. In DON-treated mesenchyme, mesenchymal cross-sectional area did not change significantly between culture times, indicating a lack of mesenchymal expansion. These results suggest that because the mesenchyme is unable to expand the positions of mesenchymal cells are maintained after division. Thus, in DON-treated mesenchyme, patterns of cell distribution are determined by differential mitotic activity. In a companion study, we present evidence that incubation with DON results in decreased amounts of HA in the cranial mesenchyme (Morris-Wiman and Brinkley, 1990). The patterns of cell distribution in DON-treated mesenchyme, therefore, may be the result of an HA-deficient extracellular matrix that is unable to initiate mesenchymal expansion.

The effects of DON treatment on neural fold morphogenesis are reflected not only in the abnormal patterning of cells within the cranial mesenchyme, but also in the reduction of mesenchymal cell numbers and in the

amplification of vascular growth into the mesenchyme. Decreased mitotic rates and increased invasion of the mesenchyme by blood vessels may also be the result of DON's interference with HA synthesis. The presence of HA is believed to facilitate mitotic activity (Toole and Gross, 1971; Tomida et al., 1974; Underhill and Keller, 1976), and endothelial cells forming new vessels avoid areas rich in HA (Feinberg and Beebe, 1983). Rat embryos cultured in *Streptomyces* hyaluronidase, an enzyme that specifically degrades HA (Ohya and Kaneko, 1970), also have reduced numbers of mesenchymal and neuroepithelial cells (Morriss-Kay et al., 1986). Enlarged, dilated blood vessels are characteristic of chick embryos injected with *Streptomyces* hyaluronidase (Schoenwolf and Fisher, 1983).

DON treatment has a major effect on the organization and behavior of the neuroepithelial and surface ectodermal layers: NE and SE cells are more rounded in treated embryos; the SE does not undergo normal flattening in treated folds; and the formation of bilaminar neural folds is abnormal. These effects may be the result of the altered composition of the basal laminae in DON-treated folds. The thickening of the neuroepithelium and the conversion of convex folds to planar form are dependent on the presence of glycosaminoglycans in the form of proteoglycans in the basal lamina (Morriss-Kay and Crutch, 1982). The defects observed in the neuroepithelium and surface ectoderm may also originate from alterations in cell–cell interactions resulting from DON's interference with the glycosylation of cell-surface receptors.

We previously proposed a model in which the initial elevation of the neural folds is produced by the expansion of the HA-rich extracellular matrix in the central regions of the cranial mesenchyme which pushes the folds medially (Morris-Wiman and Brinkley, 1989). Interference with HA synthesis would result in a failure of the fold to elevate by preventing mesenchymal expansion. Incubation with DON to block HA synthesis produces folds with abnormal patterns of mesenchymal cell and HA distribution (Morris-Wiman and Brinkley, 1990) and a decreased fold cross-sectional area. As predicted by our model, these folds fail to elevate. However, because of the pleiotropic effects of DON, we can not conclude that this failure is due entirely to a defect in mesenchymal expansion or that the lack of mesenchymal expansion results from interference with the synthesis of HA. A decreased number of mesenchymal cells, as is observed in DON-treated embryos, might also be expected to cause neural tube defects, as several studies have shown (Marin-Padilla,1966; Geelen,1973; Morriss,1973). The advent of methods that will permit the perturbation of specific extracellular matrix components in a circumscribed region of the fold is needed before we can definitely establish the roles of mesenchymal expansion and HA in neural fold elevation.

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