

The effects of *Streptomyces* hyaluronidase on tissue organization and cell cycle time in rat embryos

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SUMMARY

Day 9 rat embryos (late presomite stage with cranial neural plate or very early neural folds) were cultured for various periods of time from 6–48 h in medium containing 20 TRU ml⁻¹ *Streptomyces* hyaluronidase. Exposure to the enzyme resulted in considerable reduction of mesenchymal extracellular matrix. Access of the enzyme to the embryo was confirmed by alcian blue staining which indicated considerable reduction of extracellular and cell surface hyaluronate. Cranial neurulation was retarded, but not inhibited, and migration of both neural crest and primary mesenchyme cells occurred. In general, morphology was normal at 48 h.

The major effect was on growth: embryos were smaller, with slightly reduced neuroepithelial cell number and greatly reduced mesenchymal cell number. Neuroepithelial cell cycle time was slightly prolonged, and that of the mesenchyme more than doubled. This differential effect on the growth rates of these two tissues reflects the normal distribution of hyaluronate, which is particularly abundant in the mesenchymal extracellular matrix.

INTRODUCTION

Hyaluronate (HA) appears to play a variety of roles during embryonic development: it weakens adhesive attachments (Toole, 1981; Larterra, Lark & Culp, 1982), provides hydrated spaces through which cells migrate (Hay, 1980; Pratt, Larsen & Johnston, 1975; Fisher & Solursh, 1977; Solursh, Fisher, Meier & Singley, 1979) and is sometimes associated with cell proliferation (Toole, 1981; Cohn, Cassiman & Bernfield, 1976). We suggested previously that it might also be involved in cranial neural fold elevation in rat embryos, since hyaluronate levels are higher in the cranial mesenchyme than in the posterior half of the embryo, and the volume of mesenchyme underlying the cranial neural folds increases as the folds become increasingly convex during the first stage of neurulation (Solursh & Morriss, 1977; Morriss & Solursh, 1978a). Since then, *Streptomyces* hyaluronidase has been observed to bring about neural tube defects in chick embryos (Schoenwolf & Fisher, 1983).

This study was carried out in order to examine the role of hyaluronate in neurulation and to gain insight into other aspects of the role of hyaluronate in

Key words: hyaluronidase, mesenchyme, neurulation, cell cycle time, rat embryo.

relation to cell behaviour and tissue organization in rat embryos during the period of neurulation.

MATERIALS AND METHODS

Embryo culture

Wistar strain rat embryos were explanted in Tyrode's saline on the afternoon of day 9 of pregnancy (day of positive vaginal smear = day 0). The culture medium was 100 % rat serum; other culture details as described previously (Morriss-Kay & Tuckett, 1985). 93 embryos were cultured for 48 h in control medium and in medium containing 20 TRU ml⁻¹ *Streptomyces* hyaluronidase (which specifically degrades hyaluronate: Ohya & Kaneko, 1970) as indicated in Table 1. This enzyme concentration was chosen on the basis of preliminary experiments which showed it to have removed mesenchymal extracellular matrix so that the cells were tightly packed after 24 h, without affecting embryonic viability during 48 h culture. Ten embryos were cultured for 3 h with the enzyme then washed and cultured for a further 45 h in control medium. In addition, six control and six hyaluronidase-exposed embryos were cultured for 16 h in order to examine earlier effects on neural fold structure. After culture the fetal membranes were removed and the embryos were examined and photographed. Protein content was determined on five embryos of each treatment group by colorimetry (Lowry, Rosebrough, Farr & Randall, 1951).

The enzyme solution was added to the medium before addition of the embryos so that any protease contaminants would be inactivated by the protease inhibitors and proteins present in the serum. Efficacy of the enzyme was confirmed in three ways, as follows. (1) By examination of sections containing the heart, in embryos cultured for 48 h. Cardiac jelly, which is rich in hyaluronate (Manasek, Reid, Vinson, Seyer & Johnson, 1973) was virtually absent so that myocardium and endocardium were apposed. (2) Human umbilical cord sections were incubated for 3 h with samples of culture medium taken at the start of culture, at 10 h and at 48 h. This procedure was used as a bioassay for hyaluronate-specific hyaluronidase activity of the culture medium, since the cord matrix (Wharton's jelly) is rich in hyaluronate. Alcian blue staining at pH 2.5 was considerably reduced in all sections, confirming that the enzyme was active throughout the 48 h of culture. (3) Alcian blue staining of cultured embryos, as described below.

Morphological examination and cell counting

Five embryos of each treatment group from each of three 48 h cultures and from the 16 h culture were fixed in 2.5 % cacodylate-buffered glutaraldehyde, washed in buffer and postfixed in cacodylate-buffered osmium tetroxide; they were then dehydrated in graded alcohols and embedded in Spurr resin at an orientation suitable for the desired cutting plane. Semithin (0.5–1 µm) sections were cut on a Porter–Blum ultramicrotome, mounted on glass slides and stained with equal parts of 1 % methylene blue and 1 % azure II in 1 % borax. Embryos prepared for cell cycle time determination (see below) were also examined morphologically.

The total number of cells within the forebrain and hindbrain neural epithelium and in the mesenchyme was counted in the transverse plane on 10 sections from each embryo; three embryos of each group cultured for 48 h were assessed in this way (controls, enzyme-exposed for 3 h+45 h in control medium, and enzyme-exposed for 48 h). The position of the sections was as indicated on Fig. 1A, and illustrated in Fig. 2.

Cell cycle time determination

A further 60 embryos were cultured in either hyaluronidase-supplemented or addition-free (control) rat serum for a minimum of 16 h prior to [³H]thymidine supplementation. [³H]thymidine (5 µCi ml⁻¹, specific activity 5 Ci mmol⁻¹, Amersham International) was added at 2 h intervals to different cultures and subsequently the embryos were cultured continuously in the presence of the label. The cultures were terminated by a quick but thorough wash in saline

and immersion in Bouin's aqueous fixative. Control cultures were terminated after a total culture time of 28 h (i.e. a maximum labelling time of 12 h) and hyaluronidase cultures after 36 h (maximum labelling time 20 h). The embryos were processed for autoradiography as described previously (Tuckett & Morriss-Kay, 1985).

The percentage of labelled cells within the forebrain and hindbrain neural epithelium and in the mesenchyme was determined by means of light microscopy. The data were plotted graphically and the cell cycle time was taken to be the time at which a plateau in the labelling index was obtained, i.e. the time taken for all cells capable of dividing to incorporate [^3H]thymidine.

Alcian blue staining

After 6, 16, 24 and 48 h of culture, two control and two enzyme-treated embryos were fixed in 10 % formalin in PBS with 0.5 % cetyl pyridinium chloride, embedded in paraffin wax and sectioned at 8 μm . They were stained with alcian blue 8G-X at pH 1.0 or pH 2.5 and viewed with a light microscope. Intensity of the blue staining of extracellular matrix and cell surface components was scored subjectively on a scale from strong to absent. At pH 2.5 alcian blue stains all polyanions, including glycoproteins, hyaluronate and sulphated glycosaminoglycans (GAG); at pH 1.0 only sulphated GAG stain (Lev & Spicer, 1964).

RESULTS

Embryonic viability, size and protein content

No embryos died during culture. All embryos cultured for 48 h had a strong heartbeat, although initiation of the heartbeat was slightly delayed in embryos continuously exposed to the enzyme. Typical size relationships of whole embryos from different treatment groups are illustrated in Fig. 1. The differences are reflected in their relative protein content (Table 1A).

Morphology and cellular organization

Development of control embryos was indistinguishable from the development expected *in vivo* during the same period. After 48 h, all embryos had closed cranial neural tubes regardless of the type of medium (Fig. 1). However, the process of neurulation was retarded in embryos exposed continuously to the enzyme: the cranial neural folds were less elevated than those of controls at 6 h; at 32 h they were still V-shaped in profile, while all 28 h control embryos had closed cranial neural tubes.

Somite number was comparable in 48 h-cultured embryos of all treatment groups (Table 1) and there were no major abnormalities. Examination of whole live embryos showed little difference between control and 3 h-exposed embryos except that the cranial mesenchyme cells were denser and more sharply outlined in the latter. The dense region indicating mesenchyme was smaller in extent in 48 h-exposed embryos, and the otocyst was still an open otic pit in some specimens. The cranial neural tube was closed in all specimens, and similar to that of controls in terms of overall shape.

In sections (Fig. 2), the distance between Rathke's pouch and the hindbrain was reduced in 3 h-exposed embryos. In 48 h-exposed embryos Rathke's pouch was absent and the forebrain neural epithelium had expanded backwards to within

a short distance of the hindbrain; the thin roofplate area of the hindbrain was poorly defined or absent. Blood vessels of normal size and position, containing blood cells, were present in the mesenchyme of these embryos.

At the cellular level, the clearest effect was on mesenchymal organization. In control embryos, neural crest cells were distinguishable from primary mesenchyme cells by their position (Tan & Morriss-Kay, 1985) and by their more intense staining (Nichols, 1981). In embryos exposed to hyaluronidase for the first 3 h only, neural crest cells and the most lateral primary mesenchyme cells were clumped together, while the medial mesenchyme had a similar cell:extracellular space ratio to that of controls. Embryos exposed to hyaluronidase for 48 h showed some extracellular spaces around the foregut but not elsewhere. The presence of mesenchymal cells rostral to the forebrain neural epithelium suggested that neural crest cell migration had occurred, since in normal embryos cells in this position are of midbrain neural crest origin (Tan & Morriss-Kay, 1985, 1986). Loss of matrix was not immediate: embryos fixed and sectioned after 16 h exposure retained some extracellular spaces but with a reduced volume, even though a 3 h exposure resulted in the lateral mesenchymal cells being tightly packed (Fig. 2B). In the spinal region intercellular spaces were virtually abolished, but apart from a retardation of posterior neuropore closure, morphology was normal.

Table 1. *Growth of whole embryos and tissues*

(A) *Protein determination*

	n	No. somites	av. protein/embryo (μ g)
Control embryos, 48 h (C48)	5	18–24	151.2 \pm 26.90*
HAase 3 h, +45 h (H3/C)	5	17–25	141.0 \pm 33.48
HAase 48 h (H48)	5	19–23	89.8 \pm 12.03

* Mean \pm standard deviation.

n, number of embryos.

Comparison of protein content: C48 v. H3/C: $P > 0.05$; C48 v. H48: $P < 0.0025$; H3/C v. H48: $P < 0.01$ (χ^2 test).

(B) *Cell counts in transverse sections as in Fig. 1*

	Neural epithelium		Mesenchyme
	forebrain	hindbrain	
C48	944.9 \pm 86.83*	436.3 \pm 20.47	864.5 \pm 20.89
H3/C	773.8 \pm 23.31 (81.9%)†	393.4 \pm 30.39 (90.2%)	756.2 \pm 26.95 (87.5%)
H48	568.5 \pm 14.27 (60.2%)†	313.35 \pm 16.54 (81.8%)	378.2 \pm 16.34 (43.7%)

* Mean \pm standard deviation.

† Percentages of control values for each tissue.

Comparisons of cell number by 2-way analysis of variance: forebrain and hindbrain C48 v. H3/C, C48 v. H48 and H3/C v. H48 all $P < 0.001$; mesenchyme C48 v. H3/C: $P < 0.001$; C48 v. H48 and H3/C v. H48: $P \ll 0.001$. Three embryos in each group, ten sections per embryo assessed.

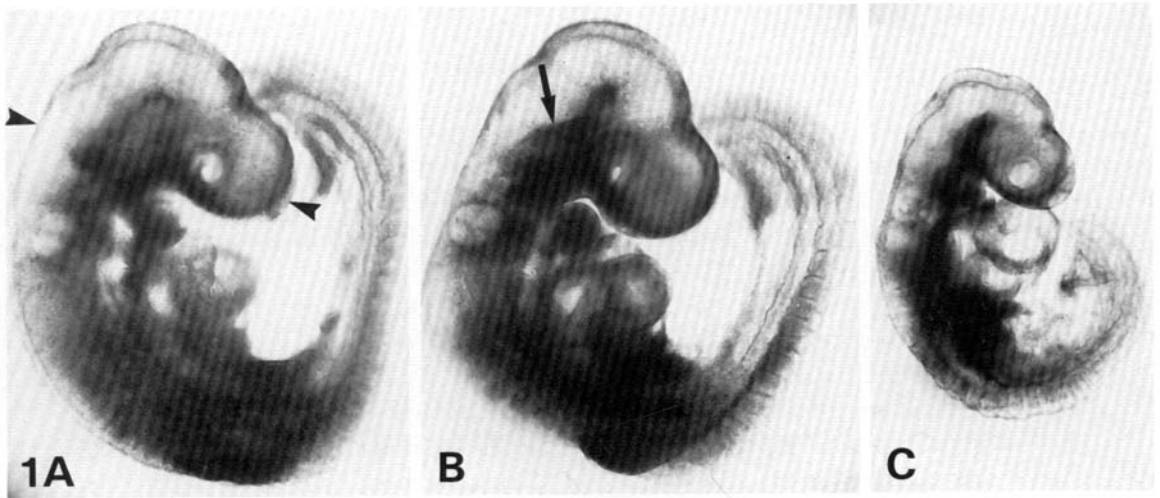


Fig. 1. Embryos cultured for 48 h in control medium (A), medium containing 20 TRU ml^{-1} *Streptomyces* hyaluronidase for 3 h followed by 45 h in control medium (B), and in medium containing hyaluronidase for 48 h (C), photographed live after removal of membranes. Arrowheads on (A) indicate the plane of sections illustrated in Fig. 2. Arrow on (B) indicates sharp demarcation of clumped mesenchyme cells (see Fig. 2B).

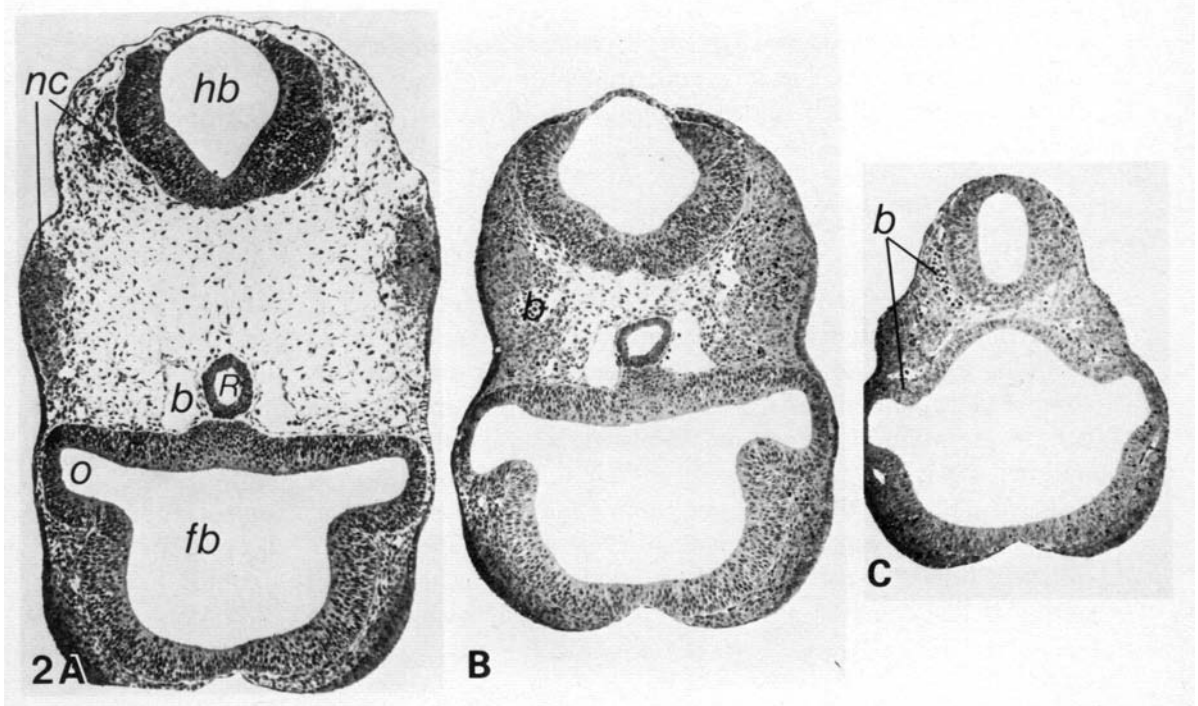


Fig. 2. Transverse sections, plane as indicated in Fig. 1A. (A–C, media as in Fig. 1.) See text for description. *b*, blood vessels; *fb*, forebrain; *hb*, hindbrain; *nc*, neural crest; *o*, optic vesicle; *R*, Rathke's pouch (not present in 48 h enzyme-treated embryos, C).

Alcian blue staining

Subjective scoring of the intensity of blue-stained material on cell surfaces and within the extracellular matrix is shown in Table 2. Stronger staining of apical neuroepithelial cell surfaces was seen at pH 2.5 than at pH 1, but apical surface staining of neuroepithelial and surface ectoderm cells was not affected by the enzyme. These observations suggest that the staining seen on these surfaces was due to the presence of glycoproteins. They will therefore not be referred to in the following comparisons.

At pH 2.5 the staining intensity of *Streptomyces* hyaluronidase-treated embryos was generally weaker or much weaker than that of controls. The only exception was the basement membrane of the surface ectoderm. The mesenchymal staining difference was less pronounced at 6 h than subsequently. Staining of *Streptomyces* hyaluronidase-treated embryos was identical at pH 2.5 and pH 1 except for some variability of endodermal and surface ectoderm basement membranes. Comparison of the staining pattern of control embryos at pH 1 with hyaluronidase-treated embryos at both pH values showed a similar pattern except for weaker mesenchymal staining of embryos exposed to the enzyme for 16 h or more. This suggests that some sulphated GAG are being removed with the hyaluronate from this tissue.

In enzyme-treated embryos cultured for 48 h the mesenchyme immediately adjacent to the foregut showed some regeneration of intercellular spaces, and some extracellular material organized as fine strands and small blobs was observed here at pH 2.5. The strands were less strongly stained than those of control embryos. Extracellular matrix staining was particularly intense in this region in controls.

Cell number and cell cycle time

Cell counts of the neural epithelium and mesenchyme were made from the sections illustrated in Fig. 1 and from other similar sections. The results (Table 1B) show that, in comparison with control embryos, neuroepithelial cell number in embryos exposed to hyaluronidase for 48 h was significantly reduced. Mesenchymal cell number was more than halved, suggesting a much greater effect on cell proliferation in this tissue. Within the neural epithelium the reduction in cell number was greater in the forebrain than in the hindbrain.

The cell number differences between 48 h enzyme-exposed and control embryos were correlated with different degrees of prolongation of the cell cycle time in different tissues. Neuroepithelial cell cycle time was increased from 6 h to 8 h, and mesenchymal cell cycle time more than doubled, from 8 h to 17 h (Fig. 3).

DISCUSSION

The association of hyaluronate with extracellular spaces is assumed to be related to its enormous capacity for water binding: from the unhydrated state it can take

Table 2. Alcian blue staining of sections of cultured embryos

	6 h						16 h						24 h						48 h					
	pH2.5		pH1.0		pH2.5		pH1.0		pH2.5		pH1.0		pH2.5		pH1.0		pH2.5		pH1.0		pH2.5		pH1.0	
	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase	Control	HAase
<i>Neural epithelium</i>																								
apical cell surfaces	+	+	±	±	+	+	±	±	+	+	±	±	+	+	±	±	+	+	±	±	+	+	±	±
basement membrane	++	+	+	+	++	+	+	+	++	+	+	+	++	+	+	+	++	+	+	+	++	+	+	+
<i>Cranial mesenchyme</i>																								
cell surfaces	++	+	+	+	++	±	+	±	++	±	+	±	++	±	+	±	++	±	+	±	++	±	+	±
extracellular matrix	++	+	+	+	++	±	+	±	++	±	+	±	++	±	+	±	++	±	+	±	++	±	+	±
<i>Surface ectoderm</i>																								
apical cell surfaces	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
basement membrane	+	+	+	+	+	+	±	±	+	+	±	±	+	+	+	+	+	+	+	+	+	+	+	±
<i>Endoderm</i>																								
apical cell surfaces	++	+	+	+	++	+	+	+	++	+	+	+	++	+	+	+	++	+	+	+	++	+	+	+
basement membrane	++	+	+	±	++	±	+	+	++	±	+	+	++	±	+/±	+	++	±	+/±	+	++	±	+	+

Key to staining intensity: ++ strong, + good, ± weak.

up sufficient water to increase its volume up to a thousand times (Ogston & Stanier, 1951). In chick embryos the production of hyaluronate-rich extracellular spaces has been associated with cell migration, both in the primary mesenchyme (Fisher & Solursh, 1977; Solursh, 1976) and in the cell-free space into which cranial neural crest cells migrate (Pratt, Larsen & Johnston, 1975).

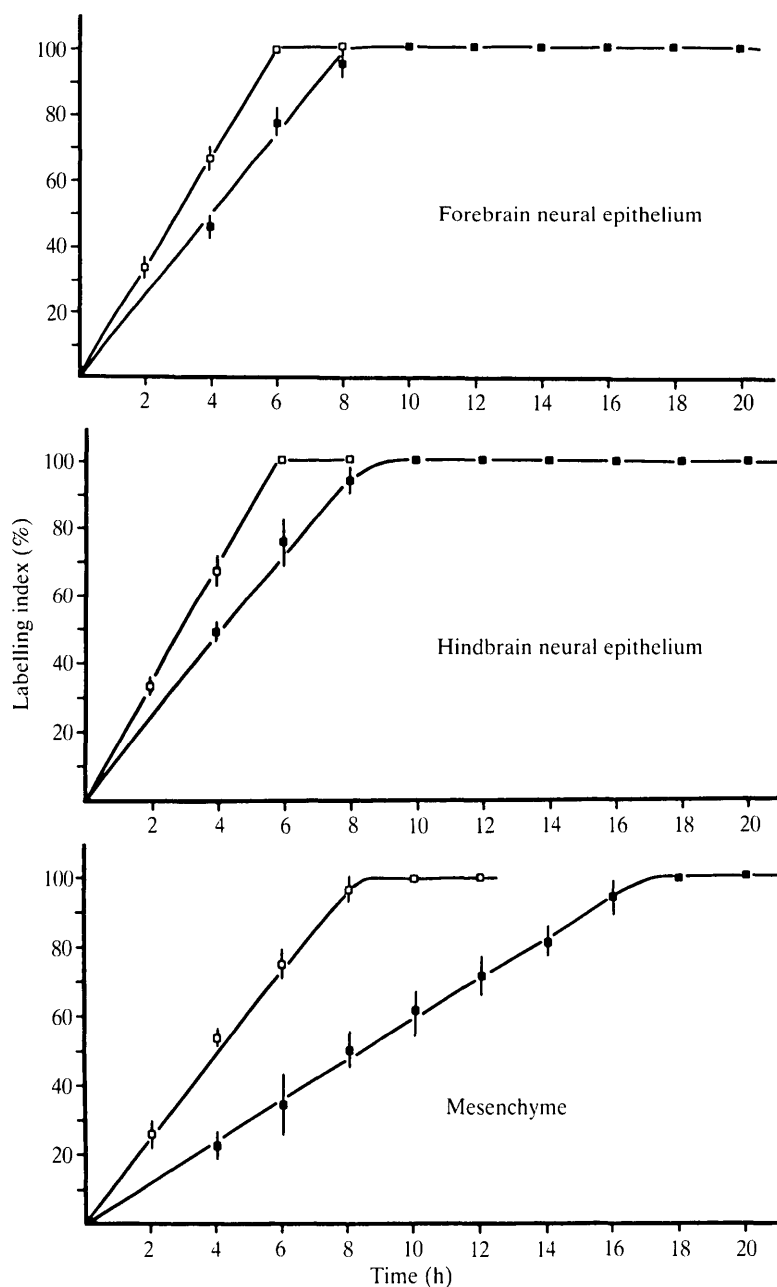


Fig. 3. Graphs illustrating the cell cycle time data for control (open boxes) and *Streptomyces hyaluronidase*-treated embryos (closed boxes).

In rat embryos during neurulation the distribution of hyaluronate-rich extracellular spaces differs from that of the chick, in that the mesenchymal cells underlying the cranial neural plate or neural folds are widely separated, whereas those elsewhere are closely packed (Morriss & Solursh, 1978a). At the early neural plate stage, even though there is relatively little cranial mesenchyme compared with later stages, the ratio of hyaluronate synthesis in the cranial and primitive streak halves of the embryo was 1.75:1 (Solursh & Morriss, 1977). As cranial neurulation progresses the volume of extracellular matrix-rich cranial mesenchyme increases; we suggested previously that this tissue might play a mechanical role in early neurulation by supporting the increasingly convex neural folds (Morriss & Solursh, 1978a,b). The observations described here indicate that although the process of cranial neurulation is retarded when the mesenchymal extracellular matrix volume is reduced, neural tube formation is not inhibited. Hyaluronate cannot therefore be regarded as essential for cranial neural tube formation in rat embryos, though it may influence timing. In contrast, treatment of chick embryos *in ovo* with *Streptomyces* hyaluronidase during neurulation resulted in a high incidence of both cranial and spinal neural tube defects (Schoenwolf & Fisher, 1983).

The observed alcian blue staining pattern (Table 2) is consistent with the interpretation that the enzyme is specifically and effectively removing hyaluronate from embryos cultured in its presence. All regions previously shown to be locations of hyaluronate (Morriss & Solursh, 1978a) stained more weakly than controls at pH 2.5; the staining pattern at pH 1 supports our previous interpretation that some hyaluronate in the mesenchymal extracellular matrix is complexed with sulphated GAG.

The presence of some pH 2.5-stained fibrillar material in the mesenchymal extracellular matrix close to the foregut basement membrane in embryos exposed to the enzyme for 48 h suggests some regeneration of hyaluronate here. This is the most internal part of the embryo, so most remote from the external source of the enzyme; particularly intense staining of the extracellular matrix of control embryos in this location suggests that it may be a region of particularly high GAG synthesis. Further evidence for this is the pattern of mesenchymal cell spacing in embryos exposed to the enzyme for 3 h followed by culture in addition-free medium for 45 h: the lateral mesenchymal cells remained clumped, whereas those more medial in position showed normal spacing; this observation suggests that spaces are not recreated around mesenchyme cells whose extracellular matrix has been removed (although this could be due to the persistence of some residual enzyme), and/or that the source of new matrix is adjacent to the foregut.

Although clumped mesenchymal cells were observed in embryos previously exposed to the enzyme for only 3 h, loss of alcian blue-stainable material from the mesenchymal extracellular matrix was not complete in embryos exposed for 6 h, and spaces were still present throughout the cranial mesenchyme after 16 h exposure. These discrepancies suggest that, while the enzyme was present in the mesenchyme by 3 h, degradation and/or removal of HA and its associated

sulphated GAG was not complete by 6 h, and loss of the water of hydration was not complete by 16 h.

In the spinal region intercellular spaces were virtually absent in embryos exposed to the enzyme for 48 h, but apart from a slight retardation of posterior neuropore closure, morphology and somite number were similar to those of control embryos. An essential role for hyaluronate in primary mesenchyme cell migration from the primitive streak therefore seems unlikely; this conclusion reflects our earlier observations of closely packed cells and only small quantities of hyaluronate in this region at the start of the neurulation period (Solorsh & Morriss, 1977; Morriss & Solorsh, 1978a). Similarly, there was no indication that neural crest cell migration was inhibited, since mesenchymal cells were observed rostral to the forebrain, and these cells are normally of midbrain neural crest origin (Tan & Morriss-Kay, 1985, 1986). These observations are consistent with those of Anderson & Meier (1982), who found that cell migration in the cranial region of the chick embryo took place when hyaluronate had been depleted by *Streptomyces* hyaluronidase, even when enzyme treatment had inhibited cranial neural tube closure.

Embryos exposed to the enzyme for 48 h were much smaller than controls, as reflected in comparisons of protein content. Cell counts and cell cycle time determinations indicated that growth was more severely retarded in the mesenchyme than in the neural epithelium. Interpretation of the effect on the mesenchyme is complicated by the observation that some sulphated GAG were removed by the enzyme along with hyaluronate. Sulphated GAG have been found to affect growth rate in a variety of cell types (Takeuchi, 1968; Sampalo, Dietrich & Filho, 1977), and cell density itself may modify the distribution and type of GAG present (Roblin, Albert, Gelb & Black, 1975; Cohn *et al.* 1976). Cohn *et al.* (1976) found that alterations in the amounts of GAG synthesized at cell densities associated with inhibition of growth of mouse 3T3 cells involved increase of sulphated GAG and decrease of HA at the cell surface. Although there may be type-specific differences in the relationship between changes in GAG synthesis and cell density, these observations support the interpretation that it is the loss of HA, rather than sulphated GAG, that is relevant to the observed increase in the mesenchymal cell cycle time. No such complication exists for the neural epithelium, where there was no evidence for sulphated GAG loss in addition to HA loss from the basement membrane, and where the cell cycle time was also prolonged.

The much greater effect on mesenchymal than neuroepithelial cell cycle time correlates directly with the much greater volume of hyaluronate-rich extracellular matrix associated with the mesenchyme than with the neural epithelium. This correlation suggests that in normal embryos the mesenchymal cell proliferation rate is strongly influenced by its hyaluronate-rich extracellular matrix, although our experiments do not indicate whether the mechanism involves a direct effect of hyaluronate or whether the role of hyaluronate is simply to space out the cells through its capacity for retaining water. Similarly, the effects of the enzyme reported here may be due to the removal of hyaluronate *per se*, or simply to the

loss of the intercellular spaces or to both. The possibility of a direct effect of hyaluronate removal is suggested by the observation that nucleotide inhibitors of hyaluronate synthetase can prevent cell division and cell rounding in culture (Prehm, 1986). An indirect effect could be mediated by a density-dependent growth inhibition response of the tightly packed mesenchymal cells (Toole, 1981), or through an effect on cell shape (Letourneau, Ray & Bernfield, 1980). Although the blood supply appeared to be normal, nutrient diffusion through the mesenchyme may be impeded by dense packing.

Within the neural epithelium the reduction in cell number was greater in the forebrain than in the hindbrain, while the effect on cell cycle time was the same in both regions. In normal rat embryos the forebrain is the only brain region to expand during cranial neurulation; cell proliferation is the same in all regions and forebrain expansion has been interpreted as the result of a continuous forward flow of the whole neural epithelium (Tuckett & Morriss-Kay, 1985). The greater effect on forebrain than hindbrain cell number observed here is more likely to be due to an effect on this forward neuroepithelial movement than to a differential effect on the different brain regions.

In summary, our observations suggest that during cranial neurulation in rat embryos, hyaluronate is not essential for cell migration and plays only a minor role in neuroepithelial morphogenesis. The major effect of hyaluronate degradation was on cell proliferation, particularly in the mesenchyme. The differential effect on mesenchymal and neuroepithelial cell proliferation reflects the normal distribution of hyaluronate, which is particularly abundant in the mesenchyme.

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