Mini-review: Hyaluronidases in early embryonic development

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In this paper I shall summarise a selection of the most relevant recent studies which indicate an involvement of the extracellular matrix and hyaluronidases in the control of developmental processes in the early embryo. I shall be concerned principally with processes taking place during morphogenesis. By this term I shall mean the cellular rearrangements which involve more or less large fields of the embryo. To a lesser extent I shall also discuss the relationship between these morphogenetic processes and cytodifferentiation, a term which I shall use to refer to the relatively irreversible change of an individual cell into a "typical" differentiated member of a histologically, functionally and molecularly distinct tissue.

Unfortunately, only a few direct investigations of the presence, distribution and activitity of hyaluronidases in early embryos are available in the literature. Most of the studies to date are mainly concerned with the presence and characterization of extracellular materials, and with the incorporation of radioactive precursors into these, but very few indeed have embarked onto a detailed analysis of the turnover of these compounds during the early stages of embryonic development. Much of the evidence I shall be considering will perforce be indirect.

Before embarking on this discussion, it is important to consider what hyaluronidase is. Far from being a single enzyme, the name is applied to any enzyme activity which cleaves the glycosidic bonds of hyaluronic acid (Merck Index, 4th ed, 1976). The Enzyme Commission currently recognizes at least four distinct enzymes with "hyaluronidase" activity: E.C.3.2.1.35, E.C.3.2.1.36, E.C.4.2.2.1 and E.C.4.2.99.1 (Florkin & Stotz, 1973). Furthermore, the existence of many different hyaluronidases has been demonstrated in many systems. There are different hyaluronidases in the adult frog from those of the tadpole (Lipson et al, 1971), and different activities associated with fibroblast cell surfaces from those in the interior of the same cells (Orkin & Toole, 1980a,b). Antibodies raised against rat urinary (renal) hyaluronidase do not cross-react with hyaluronidase of testicular origin and vice-versa (Law & Rowen, 1978, 1979, 1981). Tadpole tail fin-derived hyaluronidase shows a preference for hyaluronate as a substrate over chondroitin

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4-sulphate, and is unable to degrade chondroitin 6-sulphate or heparan. Testicular hyaluronidase, on the other hand, can cleave both forms of chondroitin sulphate as well as hyaluronate, and bacterial hyaluronidase cleaves chondroitin sulphate in preference to hyaluronate (Silbert & DeLuca, 1970). In the absence of hydrolysing enzymes, hyaluronate and chondroitin sulphates are relatively stable in embryos (Derby & Pintar, 1978).

With these provisos, we can now begin a discussion on the involvement of hyaluronidases in the regulation of early developmental processes in the vertebrate embryo.

1. Hyaluronidase activity during morphogenesis

(a) Non-malignant invasion in morphogenesis

Of particular interest to the clinician as well as the embryologist is the problem of invasion. For the oncologist, this phenomenon is associated with metastasis and the spatial spread of malignant tissues into surrounding areas. For the embryologist, non-malignant invasion is associated with normal morphogenetic events at various times in the development of embryos, when one tissue displaces another (see Mareel, 1979 and Vakaet *et al*, 1980 for recent reviews). The best examples of this process in embryonic development are perhaps the implantation of the mammalian blastocyst into the maternal endometrium, and the process of the formation of the definitive (gut) endoderm (Stern & Ireland, 1981; Bellairs *et al*, 1981), where the ectodermally-derived endoderm tissue displaces and inserts into the existing endodermal layer (hypoblast). Other examples include the migration of neural crest cells, initially into a relatively cell-free but glycosaminoglycan-rich matrix, and later some of them may move into the mass of mesenchymal cells forming the somites (Noden, 1978). At the level of single cells, fertilization itself could be considered as an invasive process, where the egg is "invaded" by the sperm.

As tissues and cells are frequently surrounded by extracellular materials such as basal laminae containing glycosaminoglycans and proteoglycans, invasive ability, whether malignant or not, should be accompanied by an ability of the invading cells or tissues to destroy this matrix. Thus one would expect hyaluronidase activity to be high in invading tissues in cancer and in non-malignant invasion during embryonic development. Experimental evidence confirms this: Kolaova (1977) has demonstrated high levels of hyaluronidase activity in patients with malignant tumours (see also Coman, 1953 and references

therein, and Bok, 1979, 1980, 1981). In non-malignant tissues, it is well known that the placenta and the trophoblast of the blastocyst at implantation (Salkie & Lambert, 1975; Salkie & Hannah, 1977; Yamada et al, 1977 and Bok, 1980) and sperm heads (Gould & Bernstein, 1975; Morton, 1975 and Dunbar et al, 1976) also contain high levels of this enzyme. Toole (1976) (see also Weston, 1982) has reported that about the time when somites become invaded by neural crest cells there is an increase in hyaluronidase activity and a decrease in hyaluronate, leading to coalescence of cells into dorsal root ganglia. It has also been shown (see Weston, 1982) that enzymatic removal of hyaluronate prevents the emigration of crest cells from the mesenchephalic neural folds.

As an area of de-epithelialized cells which are capable of invading another tissue (the hypoblast), and having a relatively high mitotic index (Stern, 1979), the primitive streak of the chick embryo is reminiscent of invasive tumours of epithelial origin. Local hydrolysis by mesoderm of the basal lamina adjacent to the ectoderm may be involved in aiding the locomotion of the former tissue out of the primitive streak and may be the physiological basis of epithelial-mesenchymal inductive interactions. Martinez-Palomo's (1970) review (see his Fig. 8) clearly shows that the surface of mesoderm cells in chick gastrulae stains much less intensely with Ruthenium Red than the overlying ectoderm. The figures in the papers by Morriss & Solursh (1978), Vanroelen et al (1980a,b,c) and Vakaet et al (1980) (see also Manasek, 1975 and Bernfield, 1981) also show much less glycosaminoglycan matrix surrounding mesoderm cells than that around the ectoderm in rat and chick embryos at primitive streak stages. These observations are consistent with the notion that mesoderm cells are capable of hydrolysing the immediately adjacent matrix, perhaps by the action of a hyaluronidase.

Eisen & Gross (1965) have clearly shown that the mesoderm contains much higher levels of hyaluronidase activity than the overlying epithelium in amphibian tail fins. More recently, Smith & Bernfield (1982) have clearly demonstrated that mesenchyme can degrade the glycosaminoglycan-rich basement membrane associated with the epithelium in salivary gland in vitro. Taken together, these findings strongly suggest that, in developing systems, mesenchymal tissues in general are characterized by their capacity to degrade extracellular matrix, indicating elevated hyaluronidase activity levels.

(b) Hyaluronidase and morphogenetic cell movements

Toole (1972, 1981) suggested that hyaluronate production accompanied mesenchymal cell movements in the leg and wing buds of chick embryos at the onset of cartilage differentiation, and that its removal is necessary for their differentiation (see

next section). Toole (1973) also found that if hyaluronate was administered to presumptive cartilage cells in culture they were induced to migrate. If hyaluronate was removed by the use of hyaluronidase, migration was inhibited and the cells underwent cytodifferentiation. Thus it would appear that in this system at least, hyaluronate may be involved in cell migration and its removal important for the cessation of this activity.

In the early chick embryo hyaluronate is found between the epiblast and the endoderm (Johnston & Comar, 1957; Martinez-Palomo, 1970; Manasek, 1975; Solursh, 1976; Sanders, 1979; Wakely & England, 1979; Vanroelen *et al*, 1980 a,b,c & Vakaet *et al*, 1980). There, it may serve a function of opening up the space between the two layers for the forming mesoderm to migrate into (see Bellairs, 1982). It has been suggested that it is the hygroscopic nature of hyaluronate which is responsible for this effect. The apparent lack of a basement membrane in regions immediately adjacent to the primitive streak, which are already in contact with the advancing mesoderm (see figures in Vanroelen *et al*, 1980a,b,c) might suggest that the mesoderm removes the glycosaminoglycan-rich matrix as it advances.

(c) Hyaluronate and hyaluronidases in pattern formation

Very little evidence is available about the possible involvement of either hyaluronic acid or hyaluronidases in the specification of positional information in developing systems. However, an interesting study by Kosher and collaborators (1981) has revealed that there exists a gradient of hyaluronate concentration in the developing chick limb bud. This gradient has its highest point at the distal tip of the limb bud, where the Apical Ectodermal Ridge (A.E.R.) is found, decreasing proximally. Such a gradient could be generated if the distal tip of the limb is producing hyaluronate as it grows, whilst a hyaluronidase present in all regions of the limb bud is responsible for its removal. Although nothing is known at present about the importance of this gradient in limb morphogenesis, the possibility that it may be involved in the specification of proximo-distal positional information is attractive.

(d) Folding and branching morphogenesis

Bernfield *et al* (1973) suggested a model for epithelial organ formation by branching morphogenesis involving localized synthesis and removal of the glycosaminoglycan matrix adjacent to the epithelium. This model was originally proposed to account for salivary gland morphogenesis, but it might equally well fit other similar folding, bending or branching morphogenetic events such as the formation and closure of the neural tube or lens morphogenesis. In fact, Schoenwolf & Fisher (1983) have recently reported that, in chick embryos, hyaluronidase treatment resulted in neural tube closure defects in 60-94% of cases.

In the case of salivary gland epithelium morphogenesis, a glycosaminoglycan matrix degrading function has been assigned to the mesenchyme surrounding the presumptive gland epithelium (Bernfield, 1981; Bernfield & Banerjee, 1982; Smith & Bernfield, 1982). The same situation may apply to the morphogenesis of the lung, mammary gland and other organs which undergo folding or branching morphogenesis surrounded by a mesenchymal matrix. Bernfield (1981) has demonstrated a hyaluronidase activity which is most effective at neutral pH and which increases during salivary gland morphogenesis to maximal at the period of most rapid branching, to low levels again when branching has markedly slowed.

Recent evidence has implicated hyaluronate in vasculogenesis. Feinberg & Beebe (1983) have found that exogenous hyaluronate inhibits blood vessel formation in the chick wing bud, perhaps indicating that a hyaluronidase activity may be involved in normal vasculogenesis.

(e) Hyaluronidase and ion transport in morphogenesis

Embryonic epithelia such as the epiblast of the chick embryo are transporting epithelia in the physiological sense. They pump ions such as sodium into the underlying space generating trans-epithelial voltages (Jaffe & Stern, 1979; Stern & MacKenzie, 1983; Stern, 1984), and have the asymmetric distribution of their intercellular junctions and basal lamina (Stern & MacKenzie, 1983) characteristic of transporting tissues (Ziegler, 1977).

The basal lamina underlying embryonic transporting epithelia such as the epiblast of the early chick embryo may in part serve the function of a selective permeability barrier to the flow of solutes such as some ions and sugars into and across the epithelium. The intensely negative electrostatic field generated by polyanionic matrices of hyaluronate can act as a significant barrier to negatively charged ions (Scott & Harbinson, 1968).

The conformation of hyaluronic acid matrices is sensitive to pH and to the concentration of ions such as sodium, lithium, calcium and magnesium, which affect its viscosity, circular dichroism and other properties (Phillips, 1970; Mathews & Decker, 1977; Chakrabarti, 1977). Hyaluronidase activity itself is sensitive to the ionic strength and pH of the surrounding environment (Gorham et al, 1975; Doak & Zahler, 1979; Gacesa et al, 1979). The effect is partly due to the change in conformation of the hyaluronic acid substrate (Doak & Zahler, 1979), but the enzyme itself is also affected, especially by pH (Gorham et al, 1975).

In more extensively studied transporting epithelia such as frog

skin it has been shown that some stimulators of the Sodium/Potassium pump such as vasopressin also stimulate the secretion of hyaluronidase (Ziegler, 1977).

A number of investigators have reported that hyaluronic acid matrices can act as mechano-electrical transducers (i.e. piezoelectric, converting mechanical energy into a voltage) (Jensen et al, 1954; Balasz & Gibbs, 1970; Barrett, 1975, 1976). Some of these results have been challenged as artefacts (Comper, 1977) when obtained in capillary tubes in the laboratory, but the attractive possibility remains, until further experiments are conducted, that hyaluronate matrices in situ, especially basal laminae, may at least in part be responsible for the known electrophysiological effects of changes in hydrostatic pressure applied to one or the other side of a sheet of cells (Ziegler, 1977).

The properties of hyaluronate matrices and hyaluronidase just described may act as important modulators of the concentration and distribution of ions around transporting epithelia in embryonic systems such as the epiblast in the early chick embryo. The possible action of the mesoderm which I have discussed above, in locally hydrolysing the overlying basal lamina by secretion of hyaluronidase, may be critical in effecting a fine control on the selective permeability of the epithelium, thus controlling to some extent the general physiological conditions in the milieu interieur of the embryo (Stern, 1984).

2. Hyaluronidase activity in the early chick embryo

Some preliminary experiments were performed on early chick embryos at gastrula (primitive streak) and somite stages. In a first series of experiments, pieces of epiblast (ectoderm), hypoblast, mesoderm and primitive streak dissected from chick gastrulae were cultured for 24-48 hours on basal laminae obtained from fresh human placentae (for method see Stern, 1981). The epiblast and hypoblast pieces spread evenly over the glycosaminoglycan-containing substrate, forming a sheet which resembled the structure of these tissues in the embryo (Fig. 1a). Mesoderm and primitive streak pieces, on the other hand, penetrated deep into the interior of the substrate, where they blebbed as is characteristic for poorly attached cells (Fig. 1b). This result suggests that there is a fundamental difference in the behaviour of mesodermal tissues from the others with respect to their ability to spread on glycosaminoglycan-rich substrates.

In a second series of experiments an antibody against bovine testicular hyaluronidase was raised in rabbits (see Law & Rowen, 1978, 1979, 1981). Each of 3 female rabbits were injected 4

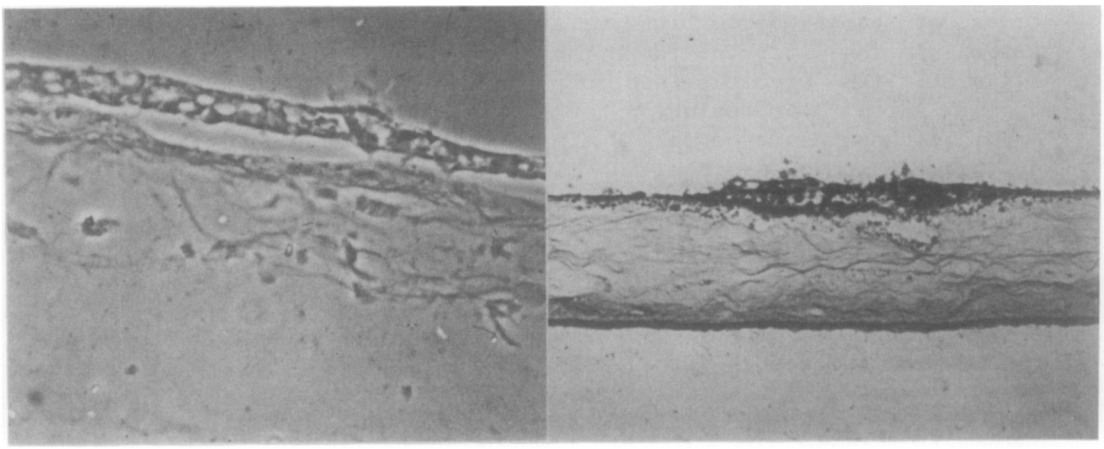

Fig. 1. A piece of stage 2 chick epiblast which has been explanted onto a glycosaminoglycan-rich substrate purified from human placenta spreads as a cohesive epithelial sheet (Fig. 1a), whereas lateral mesoderm dissected from a stage 5 embryo partly digests the substrate and expands more as individual cells. For further explanation see text. x120

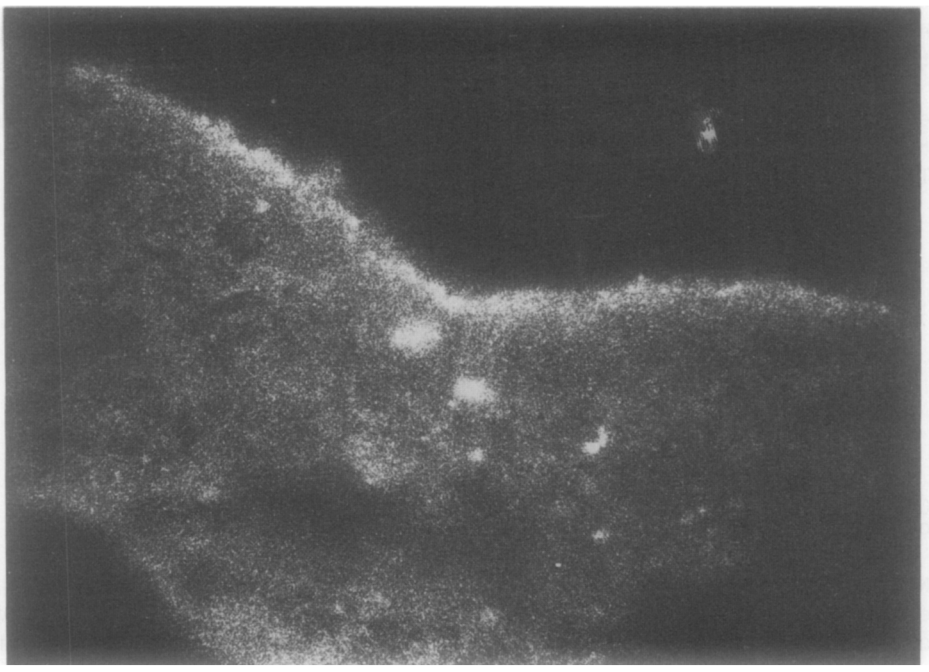

Fig. 2. Section perpendicular to the axis of the primitive streak of a stage 4 chick embryo visualized by indirect immunofluorescence using an antibody against bovine testicular hyaluronidase. The dorsal aspect of the groove of the primitive streak shows the most fluorescence. x400

times at three-weekly intervals with testicular hyaluronidase (Sigma), the first injection containing Freund's complete adjuvant. 8μm cryostat sections of chick embryos were first washed in a 0.1M solution of l-Lysine to reduce non-specific binding, then incubated for 30 min in the immune serum, followed by a 30 min incubation in fluorescein-conjugated goat anti-rabbit immunoglobulin serum. The sections were examined in a fluorescence microscope. Control sections obtained from the same embryos were incubated in pre-immune serum from the same rabbits instead of the immune serum. The only region of the sections examined which showed a clear localisation of fluorescence which differed from the pattern observed in the controls was the groove of the primitive streak at gastrulation (Fig. 2). The mesoderm at gastrulation as well as the neural tube, somite and segmental plate mesoderm at the later stage showed no detectable fluorescence.

In a third series of experiments, a series of tissues were dissected from stage 3-13 embryos to assay their hyaluronidase activity. The epiblast, hypoblast and primitive streak were explanted from stage 3 embryos, and the lateral plate mesoderm from stage 5 embryos. Somites (including adjacent neural tube) and segmental plate mesoderm (with its adjacent neural tube) were obtained from stage 11-13 embryos. The samples were pooled in saline (each sample contained material from about 25 embryos) and homogenised mechanically through a flamed Pasteur pipette. They were then assayed for hyaluronidase activity following the method of Gould & Bernstein (1975). The absorbance of solutions of known concentrations of N-acetyl glucosamine were measured by the same assay, and the relationship was found to be linear within the range of activities shown by the tissues. The following table shows the results obtained:

STAGE	TISSUE	ACTIVITY
3	epiblast	43
	hypoblast	24
	streak	190
5	mesoderm	131
11-13	somites	92
	seg. plate	48

In the above table, hyaluronidase activity is expressed as picomoles of N-acetylglucosamine liberated per cell per hour

When a mixture of the cell homogenate from primitive streaks and the anti-bovine testicular hyaluronidase immune serum described above was assayed for hyaluronidase activity, it was found that the activity of the resulting mixture was lowered down to only 41 pmoles/cell/hr. This result may suggest that most but not all the hyaluronidase activity at the primitive streak is

antigenically recognized by the antibody raised against the bovine testicular enzyme.

These results show that "mesodermal" tissues such as the primitive streak, lateral plate mesoderm, somites (which are in fact epithelial but of recent mesenchymal origin) and segmental plate mesoderm have considerably higher levels of hyaluronidase activity than the other tissues studied. The results also suggest that there may be at least two distinct hyaluronidases active at these early developmental stages, one which is present primarily at the primitive streak and antigenically cross-reacts with bovine testicular hyaluronidase, whilst the remaining activity found elsewhere does not. Alternatively, the residual activity not affected by the antibody may reflect hyaluronidase not accessible to the antibody.

3. Hyaluronidase activity and cytodifferentiation

Through the work of Bryan Toole and his collaborators, a crucial role of hyaluronidases in the control of cytodifferentiation is becoming apparent. He suggests (Toole, 1972) that hyaluronate production accompanies morphogenesis and cell movements in particular, and that its removal by a hyaluronidase activity is necessary for cytodifferentiation. He has gathered considerable experimental support for this hypothesis from a variety of systems, such as amphibian metamorphosis (Polansky & Toole, 1976), chick brain development (Polansky *et al*, 1974), amphibian limb regeneration (Smith *et al*, 1975), cornea development (Toole & Trelstad, 1971), chick chondrogenesis (Toole, 1972, 1973), chick mesonephros and metanephros organogenesis (Belsky & Toole, 1983) and chick skin and muscle fibroblasts (Orkin *et al*, 1977, Orkin & Toole, 1980a,b). No indication is given, however, as to how hyaluronidases could be causing this shift into a differentiation pathway.

Hyaluronidases have also been reported to play a controlling role in the cell division cycle, which is itself important in the control cytodifferentiation. For example, Innes (1974) has shown that 0.05% hyaluronidase applied to chick embryo and mouse oral mucosa *in vitro* increases the mitotic index of this tissue and the rate of uptake of tritiated thymidine. Greenberg & Cunningham (1973) have queried earlier claims that hyaluronidase initiated DNA synthesis in mouse fibroblasts *in vitro*. The effect was shown to be due to higher molecular weight contaminants in the crude hyaluronidase preparation. Segal *et al* (1971), on the other hand, have demonstrated that the electrophoretic mobility of purified histones (and therefore presumably their conformation and binding properties) was altered when the tissue was treated with hyaluronidase. This observation may be important in relation to a possible role of hyaluronidase in the control of gene expression. This would offer an attractive mechanism for the hypothesis put forward by

Toole (see above).

A difficulty arising from the interpretation of any studies *in vitro* (and perhaps also *in vivo*) on the effects of hyaluronidases on cell growth, cell division or DNA metabolism, however, is that the enzyme alters cell shape and cell size by virtue of its extracellular material hydrolysing properties. Cell shape and degree of spreading *in vitro* have been shown to affect cell division and other cell cycle parameters (Vasiliev & Gelfand, 1977; Curtis & Seehar, 1978). The reported effects of hyaluronidase on these parameters may therefore be indirect, and caution should be exercised when interpreting such results. Nevertheless, the possibility that hyaluronidases may control cell differentiation, gene expression and the cell division cycle directly or indirectly via changes in cell shape offers a possible important link between the processes in malignant transformation and invasion on the one hand, and normal embryonic development (morphogenesis and cytodifferentiation) on the other (Bok, 1979, 1980, 1981).

4. Summary and conclusions

The foregoing discussion indicates that hyaluronidases probably play an important part in the control of development. In morphogenesis, they may be involved in epithelial-mesenchymal inductive interactions, in non-malignant invasion when one tissue displaces another in normal development, in controlling cell movements, in modulating changes of shape of cells and sheets of cells, in controlling the permeability of tissues and regulating the ionic environment within the embryo. There is also evidence indicating that hyaluronidases are involved in the initiation of cytodifferentiation pathways, perhaps via direct or indirect effects upon the cell division cycle and histone-DNA interactions. The evidence presented indicates that hyaluronidases are important repeatedly at different stages of embryonic development and differentiation, where periods of high activity follow others of reduced activity in localized regions of the embryo.

Some new results were also presented, showing the presence of different hyaluronidase activities at early stages of chick embryo development. The highest levels of hyaluronidase activity were found in the primitive streak and mesoderm.

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