Rapid Communication

Incorporation of $^{35}$S-Sulphate in Chick Blastoderms During Elongation and During Shortening of the Primitive Streak

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Summary. Chick blastoderms were cultured for 2 h in the presence of $^{35}$S-sulphate. The distribution of the grains after light microscope autoradiography was compared in blastoderms during the elongation and during the shortening of the primitive streak. A uniform labeling was observed over the cells in both groups. Accumulation of grains was present in both groups at the ventral side of the upper layer, where transmission electron microscope studies have revealed a basal lamina. An additional accumulation of grains occurred over the cells and in the extracellular spaces of the head process and of the rostral part of blastoderms with shortening primitive streaks. This positivity could be correlated with the presence of ingressing and recently ingressed notochordal cells. Treatment of the sections with chondroitinase ABC and/or HNO$_2$ before dipping in the nuclear emulsion demonstrated that at least chondroitin sulphate and N-sulphated heparan sulphate were present.

Key words: Sulphate - Glycosaminoglycan - Autoradiography - Gastrulation - Chick

Introduction

During gastrulation in the chick blastoderm, epiblast cells ingress to form the mesoblast and the definitive endoblast. This ingress begins during the elongation of the primitive streak covering stages 3 to 6 (Vakaet 1970). From stage 7 on, the anterior half of the streak starts shortening, forming the head process in the track of Hensen’s node.

Using toluidin blue staining of chick blastoderms in vivo, Vakaet (1957) observed metachromatic staining of the young head process. This staining can be attributed to an accumulation of polyanionic groups, possibly sulphates.

Johnston and Comar (1957) have studied the incorporation of $^{35}$S-sulphate in the chick blastoderm during the elongation as well as during the shortening of the primitive streak. They observed an even distribution of the label over the blastoderms during elongation of the primitive streak. During the shortening of the primitive streak, however, they found grains accumulated over the young notochord. Manasek (1970) pointed to the difference in incubation time between the two groups. The younger blastoderms were cultured in the presence of the precursor for shorter periods than the older ones. This would not allow to compare the results in both groups.

In the present investigation, we wanted to settle this discussion by culturing blastoderms during the elongation of the primitive streak and during the shortening of the primitive streak for the same period in the presence of the same concentration of $^{35}$S-sulphate. Specificity tests were carried out, using chondroitinase ABC and/or HNO$_2$. An attempt was made to correlate the differences in grain density distributions with morphogenetic events.

Materials and Methods

Incubation

Two groups of fertilized eggs were incubated at 38°C. One group was incubated for 12 h to obtain blastoderms of stage 3 (Vakaet 1970; Hamburger and Hamilton 1951). The other group was incubated for 22 h to attain blastoderms of stage 8 (Vakaet 1970) corresponding to stage 5 of Hamburger and Hamilton (1951). The blastoderms of both groups were cultured according to the method of New (1955) for two hours, on a medium containing 0.5 ml Ringer solution with 3 mg agar and 0.5 ml egg white, in the presence of 140 μCi $^{35}$S-sulphate (carrier free; The Radiochemical Centre Ltd., Amersham). A total of 72 blastoderms was studied. Controls were carried out using embryos cultured on the above medium, without the radioactive precursor.

Tissue Preparation

The blastoderms were fixed overnight at room temperature by immersion in one of the two following fixatives: (a) 96% alcohol-formaldehyde (35-40% w/v)-acetic acid at a ratio of 75/20/5 by vol. (AFA), or (b) 10% (v/v) aqueous formalin containing 0.5% (w/v) cetylpyridinium chloride, 2% (w/v) calcium chloride, in the presence of an excess of calcium carbonate (FOCA-CPC). The blastoderms were dehydrated in a graded series of alcohol, cleared in xylene and embedded in paraffin wax. Serial sections (8 μm) of each blastoderm were divided over 7 slides, so that on each slide, sections of all areas of the embryo were present.

Histochemical Procedures

After hydration, the 7 slides were treated as follows:

1. with chondroitinase ABC (Sigma Chemical Co.) according to Derby and Pintar (1978): immersion in enriched Tris buffer (Saito et al. 1968), pH 8.62 containing 2 U/ml chondroitinase ABC for 2 h at 38°C,
2. with the heat-inactivated enzyme (in a boiling water-bath for 10 min) under the same conditions as in (1),
3. with HNO$_2$ according to Inoue and Nagasawa (1976): immersion at room temperature for 90 min in a mixture of equal amounts of 5% (w/v) sodium nitrite and 33% (v/v) acetic acid,
(4) with 16.5% (v/v) acetic acid under the same conditions as in (3),
(5) consecutively with (1) and (3),
(6) consecutively with (2) and (4),
(7) untreated.

Autoradiography

The slides were dipped at 45°C in darkroom conditions, by a double immersion in Ilford L4 Nuclear Emulsion, diluted 1/1 (v/v) in distilled water. After 5 weeks of exposition at 4°C, they were processed according to the method of Caro et al. (1962). Sections of the blastoderms cultured without the radioactive precursor were also dipped in order to control chemography.

The hydrated sections were stained either (a) before dipping, according to the method of Feulgen and Rosenbeck (1924) modified by Demalsy and Callebaut (1967), or (b) after dipping using the methyl green-pyronin method (see Lison 1960). Some sections were not stained.

Results

During the elongation of the primitive streak (Fig. 1, left), a uniform grain density was present over all the cells. Grains were also observed between the epiblast and the hypoblast. Accumulation of sulphated molecules was seen on the ventral side of the epiblast (Fig. 2A–D). This accumulation was not observed at the level of the ingressing cells. Treatment with chondroitinase ABC or HNO₂ diminished the overall grain density. Treatment with chondroitinase ABC and HNO₂ consecutively, reduced the grain density more than with chondroitinase ABC or HNO₂ alone. A residual grain density persisted over the sections. Topographical differences in grain densities did not appear after these histochemical treatments.

Blastoderms during the shortening of the primitive streak (Fig. 1, right) show a uniform grain density over the cells and an accumulation of grains on the ventral side of the epiblast. Moreover, an accumulation of grains was observed in the young head process and in the rostral part of the primitive streak (Fig. 3A–D). This accumulation is localized, at least in part, extracellularly. Treatment with chondroitinase ABC and/or HNO₂ diminished the grain density but selective removal of grains could not be detected.

Differences in grain density pattern were not observed between:
(a) blastoderms fixed in AFA or in FOCA-CPC,
(b) sections stained with the Feulgen or with the methyl green-pyronin technique,
(c) unstained and stained sections,
(d) the untreated, the buffer-treated and the acetic acid-treated sections. Sections of blastoderms cultured without the radioactive precursor and processed for autoradiography, did not show grains above background values.

Discussion

Resolution in autoradiography is governed by the energy of the emitted β-rays and consequently, by their track length. Moreover, it is influenced by the thickness of the section and the thickness of the emulsion layer (Salpeter et al. 1974; Vanroelen et al. 1981). Considering these parameters, the resolution in experiments using ³⁵S-sulphate, as a precursor, is poorer than with tritiated precursors.

We can only conclude from our experiments (1) that a higher grain density can be observed over the notochordal cells if compared to the other cells in the shortening streak stage chick blastoderm, and (2) that a different grain density pattern is observed if our two groups are compared. In both groups, the sulphated materials seem to be localized, for a major part, in the extracellular spaces. This assumption is in agreement with some results of other authors. O’Hare (1973) detected the accumulation of sulphated glycosaminoglycans in the extracellular spaces between the notochordal cells after staining with alcian blue in critical electrolyte concentrations. In an ultrastructural study, Mackey (1974) observed the presence of extensive extracellular accumulations of partially fibrillar and partially amorphous materials in the same tissue. These materials might correspond to the accumulation of sulphated macromolecules detected in our study.

Manasek (1970) was right when he criticized Johnston and Comar’s (1957) experiments. Application of the precursor for different periods to blastoderms of two different age groups would make comparison unreliable. However, our results agree reasonably well with those of Johnston and Comar (1957), although we have treated both groups of blastoderms of different age in an identical way. The higher grain density over the notochordal cells may be interpreted as a marker of differentiation of pre-chordal tissue, and this before the establishment of histologically recognizable cartilaginous material. The interpretation of autoradiographic results is not always obvious. The same grain density over different tissues (e.g. in the chick blastoderm during elongation of the primitive streak) does not necessarily imply that the same products are synthesized. Indeed, the synthesis of different products with the same degree of sulphation can result in similar grain densities. The higher grain density (above the notochordal cells), due to a higher ³⁵S-sulphate concentration in the tissue, may be caused by a higher accumulation of the same or different products. These differences may concern molecular composition, molecular weight or degree of sulphation. This higher accumulation can be caused by enhanced synthesis or by lowered breakdown (enzymatically or due to the presence of complexing products such as collagen or fibronectin). Another explanation for the higher grain density may also be, that it is the visualization of a higher sulphate uptake by the notochordal cells, or a different pool size of sulphate or its metabolites (3'-phospho-adenosine 5'-phosphosulphate) used during the synthesis.

Our histochemical procedures were not able to shed further

Fig. 1. Drawing of the area pellucida of a stage 4 (left) and a stage 8 (right) chick blastoderm. The level at which photographs of transverse sections are shown, is represented.