Altered Cell Fate in LiCl-Treated Sea Urchin Embryos

CATHERINE NOCENTE-MCGRATH, ROBERT MCISAAC, AND SUSAN G. ERNST

Department of Biology, Tufts University, Medford, Massachusetts 02155

Accepted July 5, 1991

Endo16 is a lineage-specific protein expressed during embryogenesis in the sea urchin, Strongylocentrotus purpuratus. We have examined the effects of various concentrations of lithium chloride, a well-known vegetalizing agent, on Endo16 expression in whole sea urchin embryos. Our results show that treatment with LiCl causes increased steady-state levels of Endo16 transcripts. An increase in the number of endodermal cells in treated embryos demonstrates a change in cell lineage. In addition, we observed a delay in development of lithium-treated embryos that is accompanied by a delay in the expression of a late class histone H2B gene.

INTRODUCTION

The profound effects of lithium on embryogenesis have intrigued developmental biologists since its vegetalizing action on the sea urchin was first reported nearly a century ago (Herbst, 1892). Lithium is thought to affect cell fate determinations in sea urchin (reviewed in Lallier, 1964) and frog embryos (Kao et al., 1986), as well as Dicystelium development (Peters et al., 1989). Renewed interest in this subject has been sparked by the availability of sensitive lineage-specific probes and new information on the physiological effects of lithium. Recent studies have shown that lithium has specific inhibitory effects on inositol phosphate metabolism. This has led to the hypothesis that lithium affects embryogenesis by disrupting inductive interactions mediated by second messenger pathways (reviewed in Berridge et al., 1989).

In the sea urchin embryo, lithium causes an exaggeration of vegetally derived tissues in whole embryos (reviewed in Lallier, 1964; Horstadius, 1973), and the appearance of vegetally derived structures in isolated animal caps (Livingston and Wilt, 1989). The extent of vegetalization and the final morphology depend upon the concentration of lithium used, the time of exposure, and the species of sea urchin. Classical studies on the effects of lithium were limited by their reliance on morphological criteria to identify differentiated tissues. The more recent studies by Livingston and Wilt (1989) have employed molecular markers to demonstrate that exposure to lithium can evoke the expression of vegetal-specific molecules in isolated animal blastomeres.

Endo16 is a vegetal-specific marker that is expressed exclusively in endodermal tissue (Nocente-McGrath et al., 1989). The expression of this molecule can be detected in presumptive endoderm prior to the onset of gastrulation. After gastrulation, expression of Endo16 becomes progressively restricted to a subset of endoderm tissue. The present study examines the effects of lithium exposure on the temporal and spatial expression of Endo16 using quantitative hybridization analysis and indirect immunofluorescence on whole mount and single cell preparations. We have also investigated the developmental delay observed in vegetalized embryos using a late histone H2B probe as a molecular index of developmental progression.

MATERIALS AND METHODS

Animals. Adult Strongylocentrotus purpuratus were purchased from Marinus, Inc. (Long Beach, CA). Maintenance of animals, spawning, fertilization, and embryo culture were as previously described (Pittman and Ernst, 1984). Dissociation of mesenchyme blastula embryos to single cells was accomplished using standard methods (Pittman and Ernst, 1984). Embryos were pelleted, washed twice with calcium- and magnesium-free sea water (CMFSW), and resuspended in 1 M glycine, pH 7.8. Cell clumps were dispersed by gentle pipetting and the cells were washed once with CMFSW prior to application onto polylysine coated slides.

Lithium chloride vegetalization of sea urchin embryos. Embryos were exposed to lithium chloride (LiCl) continuously between 30 min and 16 hr postfertilization. Briefly, a 1-liter culture of fertilized eggs was divided equally into a control and three LiCl-treated cultures. This was accomplished by transferring 250 ml of embryos to three separate jars containing 750 ml of Milli-
pore-filtered sea water (MPFSW) supplemented with LiCl to final concentrations of 15, 30, and 60 mM. The control embryos (250 ml) were diluted to 1 liter and the cultures were allowed to develop. At 16 hr postfertilization, embryos were pelleted by spinning 5 min at 400 rpm in a Damon CRU-5000 centrifuge. The pellets were washed twice with MPFSW and resuspended in 1 liter of MPFSW to continue development.

**Northern blot analysis.** Standard molecular biology techniques were employed (Maniatis et al., 1982). Embryonic RNA was purified by the guanidine/CSIC method (Chirgwin et al., 1979), size fractionated on a 1% formaldehyde–agarose gel, and blotted to nitrocellulose. The filter was hybridized sequentially to 32P-labeled probes specific for End016 and for the late class histone H2B gene (Maxson et al., 1983). Hybridization conditions for both clones were 40% formamide, SSPE, 5X Denhardt's, 0.5% SDS at 42°C. Filters were washed in 0.2X SSC, 0.5% SDS at 65°C. Autoradiography was performed with Kodak XAR5 film and a DuPont Cronex intensifying screen at -70°C.

**Indirect immunofluorescence.** Whole mount embryos and single cells were fixed and stained as previously described (Nocente-McGrath et al., 1989), with the following modification. Affinity-purified anti-End016 antibodies were used at a 1:100 dilution in PBS. A complete description of the affinity purification will appear elsewhere (manuscript in preparation). Briefly, anti-End016 antibodies were purified by column chromatography over agarose coupled to a β-galactosidase–End016 fusion protein. Stained slides were mounted in FITC-Guard mounting medium (Testog Inc., Chicago, IL). The reaction was viewed and photographed under a Zeiss microscope equipped for epifluorescence using Kodak T-Max film at an ASA setting of 800. In addition, dissociated preparations were stained for End016 and counterstained with DAPI to determine the total number of cells in an individual field. The cells were photographed for End016 and for DAPI and three fields of approximately 400 cells each were counted for End016 staining and DAPI staining.

**RESULTS**

**Morphological Characteristics of Vegetalized Embryos**

As a prerequisite for examining the effects of LiCl exposure on End016 expression, the overt morphological characteristics of *S. purpuratus* embryos with varying degrees of vegetalization were documented. Pluteus stage embryos that had been cultured in the presence of increasing amounts of lithium chloride were fixed and photographed. Variation in the response to LiCl treatment by batches of eggs from different females was observed. The morphological profile described below was obtained in all cultures, but the specific concentration of lithium required to produce the effects fluctuated among batches of eggs collected from different females.

Untreated pluteus embryos (Fig. 1A) possess a well-developed three-part gut, a complete skeleton, and pigment cells distributed throughout the ectoderm. Embryos treated with 15 mM LiCl (Fig. 1B) display everted guts due to exogastrulation. These embryos appear nearly normal with respect to the proportions of the primary tissue layers. In contrast, embryos cultured in the presence of 30 mM LiCl exhibit a striking vegetalized morphology (Fig. 1C). These embryos appear as a three-part “snowman” shape. The largest sphere is decorated with pigment cells and encloses skeletal spicules, features characteristic of larval ectoderm. The spicules are small and abnormally shaped. The two smaller spheres are attached to the larger one by a ring of constricted cells and together appear to be a greatly enlarged gut. The smallest sphere often contains an invaginated structure. When embryos are exposed to 60 mM LiCl, they undergo cleavage which results in relatively normal blastulae, but they become increasingly disorganized as morphogenesis proceeds (data not shown). They appear as variously sized masses of cells, small clumps, and single cells by the time controls have completed embryogenesis. Pigment cells can be identified in the masses, but there is little evidence of any tissue organization. These results are in agreement with earlier reports that there is a graded response to increasing LiCl concentrations characterized by an increase in endoderm-like tissue and a concurrent decrease in ectoderm.

**Immunolocalization of End016 in Whole and Dissociated Vegetalized Embryos**

To investigate the spatial expression of End016 in vegetalized embryos, whole, fixed embryos were stained with affinity-purified anti-End016 antibodies (Fig. 2). Embryo cultures were exposed to various concentrations of LiCl and harvested at 36-hr gastrula and 84-hr pluteus stages for staining. Antibody staining was visualized with fluorescein-conjugated goat anti-rabbit secondary antibody and observed under a fluorescence microscope. As previously reported (Nocente-McGrath et al., 1989), control embryos stained intensely over the entire archenteron surface at the gastrula stage, with this staining becoming less intense and restricted primarily to the midgut by the pluteus larval stage (Figs. 2A and 2D). In all cultures exposed to LiCl, a delay in hatching and gastrulation was observed so that at 36 hr these embryos had not initiated gastrulation (Figs. 2B and 2C). The vegetal plate region of these embryos stained intensely, and the domain of reacting cells appears...
Fig. 1. Morphology of vegetalized embryos at pluteus larva stage. Cultures were exposed to various concentrations of LiCl as detailed under Materials and Methods. Embryos were fixed in 1% formaldehyde and photographed with the aid of a polarizing filter to highlight the larval skeletal system. (A) Control embryos. The three-part gut and skeletal system are well developed. Pigment cells can be seen dispersed throughout the ectoderm. (B) The 15 mM LiCl-treated embryos. Exogastrulated embryos have normal skeletons and pigment cell distribution. (C) The 30 mM LiCl-treated embryos. Snowman-shaped embryos have abnormal skeletons and asymmetric pigment cell distribution.

The Effect of LiCl on the Temporal Expression of Endo16 mRNA

The effect of LiCl on the temporal and quantitative expression of Endo16 mRNA in the developing sea urchin embryo was characterized. Figure 3 shows the results of Northern blot analysis of Endo16 expression in 15- and 37-hr control and LiCl-treated embryos. In both treated and untreated embryos, Endo16 expression is not detected at 15 hr, indicating that the gene is not activated prematurely in vegetalized embryos. At 37 hr, a stage of development when this gene is normally expressed at high levels, a concentration-dependent increase in the level of Endo16 mRNA is observed in embryos treated with LiCl. The batch of eggs used in this experiment required treatment with 60 mM LiCl to effect the completely vegetalized morphology pictured in Fig. 1C.

In the present study, a generalized morphological delay for embryos exposed to LiCl was observed. To examine the molecular characteristics of this delay, the Northern blot of control and LiCl-treated RNA at 15 and 37 hr of development was rehybridized with a probe specific for the late class histone H2B gene (LH2B) (Maxson et al., 1983b). The late histone genes are normally...
TABLE 1
FRACTION OF CELLS EXPRESSING ENDO16 PROTEIN IN NORMAL AND VEGETALIZED EMBRYOS

<table>
<thead>
<tr>
<th>[LiCl]</th>
<th>Anti-Endo16 stained cells at the m. blastula stage</th>
<th>Relative increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>23%</td>
<td>-</td>
</tr>
<tr>
<td>15 mM</td>
<td>26%</td>
<td>1.13</td>
</tr>
<tr>
<td>30 mM</td>
<td>36%</td>
<td>1.52</td>
</tr>
<tr>
<td>60 mM</td>
<td>40%</td>
<td>1.74</td>
</tr>
</tbody>
</table>

transcribed at very low levels during cleavage stage and the mRNAs rise sharply in abundance during the period from 14–16 hr postfertilization, a time when the abundance of the early histone mRNAs declines rapidly. While Endo16 levels are induced in LiCl-treated cultures, LH2B expression is markedly lower (Fig. 3). Later in development, equivalent levels of LH2B histone are found in control and LiCl-treated embryos (data not shown). Comparison of Endo16 and LH2B signals reveals a differential response to LiCl in the expression of a lineage-restricted gene (Endo16) and a temporally regulated, nonlineage-restricted gene (LH2B).

FIG. 2. Immunolocalization of Endo16 in vegetalized embryos. Cultures were exposed to various concentrations of LiCl as detailed under Materials and Methods. Embryos were collected at 28 hr (late mesenchyme blastula, panel G), 36 hr (midgastrula stage; A, B, and C) and at 84 hr (pluteus larva stage; D, E, and F). Whole mounts were fixed and stained with anti-Endo16 antibodies as described under Materials and Methods. (A, D) Control embryos. Entire archenteron appears to stain at the earlier stage (A). Intensity of staining decreases as development proceeds (D). (B, E) The 15 mM LiCl-treated embryos. Developmental program is delayed in treated embryos as evidenced by delayed gastrulation (B, C). The vegetal plate region stains intensely, characteristic of the mesenchyme blastula stage seen in (G). Everted guts at the pluteus stage (E) also stain intensely. (C, F) The 30 mM LiCl-treated embryos. As in (B), gastrulation is also delayed in these embryos. Note the flattened shape of the embryo in (C) and the larger sector of stained cells in comparison to control embryo at the same morphological stage (G). Midpiece and "head" of the pluteus stage vegetalized embryo react with the anti-Endo16 antibodies, revealing the great expansion of endodermal tissue in these embryos.

FIG. 3. The effect of LiCl on Endo16 transcript levels. RNA isolated from control and LiCl-treated embryos was size fractionated on a 2.2 M formaldehyde-1% agarose gel and blotted to nitrocellulose. The Endo16 and late H2B messages were detected by hybridizing to the filter 32P-labeled cloned fragments of the respective genes. RNA isolated 15 hr postfertilization was loaded in lanes 1–4, 37 hr postfertilization in lanes 5–8. The samples (5 μg each) were loaded in the following order: control (lanes 1, 5), 15 mM LiCl (lanes 2, 6), 30 mM LiCl (lanes 3, 7), 60 mM LiCl (lanes 4, 8). Endo16 mRNA expression was not observed until posthatching (18 hr) and was present at much higher levels after LiCl exposure. Late class histone gene expression was retarded in vegetalized embryos.

DISCUSSION

Both classical and modern studies have shown that exposure to lithium during the early stages of development causes cells to adopt a more vegetal fate, and at the molecular level, to express vegetal markers that they would not otherwise synthesize. Since Endo16 was found to be expressed exclusively in cells of a vegetal lineage (endoderm), it was of interest to examine the effects of LiCl exposure on Endo16 expression. Classical studies on whole embryos treated with lithium reported a graded response, and the data presented here for S. purpuratus support these observations. Furthermore, these data show that this response occurs at the molecular as well as the morphological level. These results are consistent with the work of Livingston and Wilt (1989) detailing a LiCl dose-dependent response for the development of vegetal markers in isolated animal blastomere cultures. The indirect immunofluorescence staining results provide molecular confirmation of the identification of endodermal tissue that relied on morphological traits in the older literature, clearly demonstrating a change in cell fate resulting from exposure to LiCl. In addition, the whole mount and single cell staining experiments demonstrate that anti-Endo16 antibodies detect an increase in presumptive endodermal tissue even prior to the onset of gastrulation. This increase could cause a mechanical disruption of gastrulation that might contribute to the exogastrula morphology exhibited by the lithium-treated embryos.

The developmental delay in lithium-vegetalized embryos that we observed has previously been reported and is probably directly related to a slowing of cleavage rates (Hagstrom, 1963). Our results demonstrate that these effects are accompanied by a delay in histone gene class "switching" that normally occurs during development (see Maxon et al., 1983a for review). This result is somewhat surprising since the transition from early to late class histone gene expression is known to be inde-
pendent of cell–cell contact (Arceci and Gross, 1980; Brookbank, 1980), cell division (Brookbank, 1978; Harrison and Wilt, 1982), or cell lineage (Angerer et al., 1985). Clearly, lithium exerts multiple and complex effects on developing embryos. The use of sensitive lineage-specific markers, such as Endo16, may help to further dissect this fascinating phenomenon.

We thank L. Angerer and R. Maxson for the histone LHZB probe and John McGrath for critical reading of the manuscript. Supported by a R.A.W. Career Advancement Award from the NSF and a Biomedical Research Support Grant to S.G.E.

REFERENCES


