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Appearance of New Glycoproteins in Methoxychlor-Exposed Sea Urchin Gastrulae

J. M. Mwatibo, J. D. Green

Department of Cell Biology and Anatomy, Louisiana State University Medical Center, New Orleans, LA 70112, USA

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Environmental pollutants and teratogens are known to induce alterations in cell proliferation, cell migration, cell-cell and cell-matrix interaction and extracellular matrix composition. Such alterations have profound effects on early embryonic development. We have been investigating the effects of environmental pollutants on early development, using the sea urchin as a model. Sea urchins have a well-characterized pattern of rapid and synchronous development horn zygote through pluteus, which facilitates the collection of identical stages for cellular and biochemical analysis. Our previous studies showed that acute exposure of sea urchin zygotes at 10 min post-insemination (PI) to methoxychlor (MXC), a widely used pesticide, resulted in development of stunted archenterons and incomplete spicules (Green *et al.* 1997). Since gastrulation involves a number of cell-cell and cell-matrix interactions, the time of onset and the nature of the abnormalities caused by MXC exposure suggested that MXC interfered at some level with these interactions or altered the blastocoelic molecular cues. These cell-surface molecules (glycoproteins) are important as guidance cues or attachment substrata for the migratory mesenchyme cells.

The blastocoelic extracellular matrix (ECM) is composed mainly of glycoproteins and different proteoglycans (Heifetz and Lennarz 1979; Solursh and Katow 1982). Components of the ECM are closely regulated in their synthesis and are considered crucial for normal gastrulation to take place (Fink and McClay 1985). In recent years, lectins have been utilized to study the distribution of carbohydrate-containing cell-surface receptors on adult and embryonic cells. Our aim, therefore, was to use lectins to examine the expression of glycoproteins during gastrulation of MXC-exposed embryos. Since concanavalin-A (Con-A) and wheat germ agglutinin (WGA) are the most widely used lectins and their pattern of staining in the sea urchin is well characterized (Katow and Solursh 1982; Spiegel and Burger 1982; DeSimone and Spiegel 1986a,b), we chose them for use in this study to determine the effect of MXC on expression of glycoproteins at gastrulation.

Correspondence to: J. D. Green

MATERIALS AND METHODS

Sea *urchins, Strongylocentrotus purpuratus,* were purchased from Kim Siewers (Santa Cruz, CA) and maintained in an aerated and refrigerated laboratory aquarium at 14-15°C. Gamete shedding was induced by intracoelomic injection of 0.55*M* KCl and gametes were collected in artificial sea water (ASW). ASW was prepared according to Marine Biological Laboratory formulae (Cavanaugh 1956) and buffered with 10 m*M* TAPS (tris[hydroxy-methyllmethyl aminopropane sulfonic acid) to a final pH of 8.0 (Green *et al.* 1990). Semen was pipetted "dry" from the gonopores of males and stored undiluted at 4° C until use. Before use, eggs were washed in three changes of ASW to remove debris and coelomic fluid from the suspension. A 2% suspension of eggs was fertilized by the addition of semen diluted to effect 95 to 100% fertilization. After insemination and swirling of the egg-sperm mixture to thoroughly mix the gametes, the fertilized eggs were allowed to settle. The overlying ASW was aspirated to remove excess sperm and the embryos were resuspended in fresh ASW. Fertilized eggs elevate fertilization envelopes (FE) which harden before 10 min by an ovoperoxidase-dependent thiol cross-linking reaction (Foerder and Shapiro 1977). Elevated FE were counted to determine the rate of fertilization and only egg batches that achieved 95% or more fertilization were used for experiments. Normal first cleavage (ie., division into two symmetrical blastomeres) was monitored so as to check for polyspermy. Any batches of eggs which did not achieve at least 95% normal cleavage were discarded. The embryos were allowed to develop at 15° C with gentle stirring at 60 rotations per minute using motor-driven paddles. With these culture conditions, synchronous division and development of embryos was obtained. Development was monitored by periodic microscopic examination.

Methoxychlor (Grade I, 98%; Sigma) is relatively insoluble in water. Its solubility is approximately 0.1 parts per million (ppm) at 25° C. (Richardson and Miller 1960). Therefore, it was dissolved in an organic solvent, dimethyl stioxide (DMSO; Sigma) immediately before use. The final concentration of DMSO in sea water (0.5%) was shown previously not to interfere with normal development (Green *et al.* 1997). The dissolution of MXC did not change the pH (8.0) of 0.5% DMSO/ASW. At 10 min PI, zygotes were exposed to 6.25 ppm MXC for 30 min after which MXC was diluted 500-fold using 0.5% DMSO/ASW. From our preliminary dose-response experiments (Green *et al.* 1997), we chose this dosage for further study, as it interfered with normal gastrulation, but did not abolish it. The embryos were allowed to continue developing until gastrula stage (36 hr). The pH of the media (0.5% DMSO/ASW) was periodically checked to ensure it had remained stable (8.0). This is important because pH shifts (especially pH decreases) have been shown to induce developmental defects (Pagano *et al.* 1985). Small aliquots of gastrulae (36 hr) were fixed with 10% formalin and photographs were taken.

In addition, aliquots (1.5 mL) were taken from the embryo cultures at gastrula stage. Samples were homogenized in reducing sodium dodecyl sulfate (SDS) buffer and heated in boiling water bath for 5 min before loading onto 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Equivalent amounts of protein (30 µg/lane),

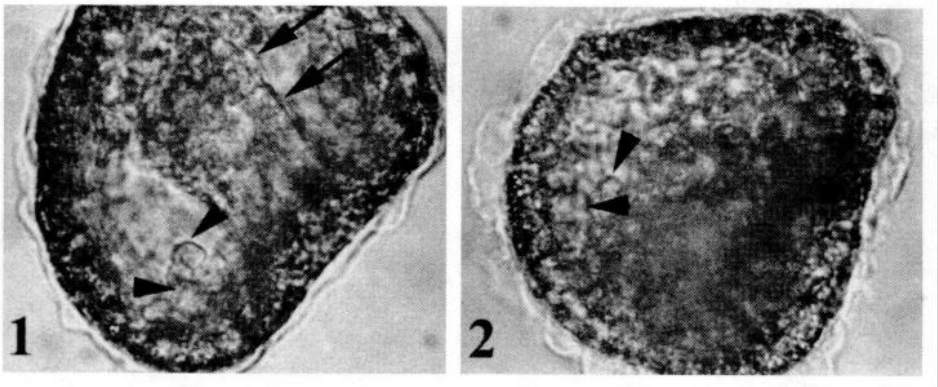


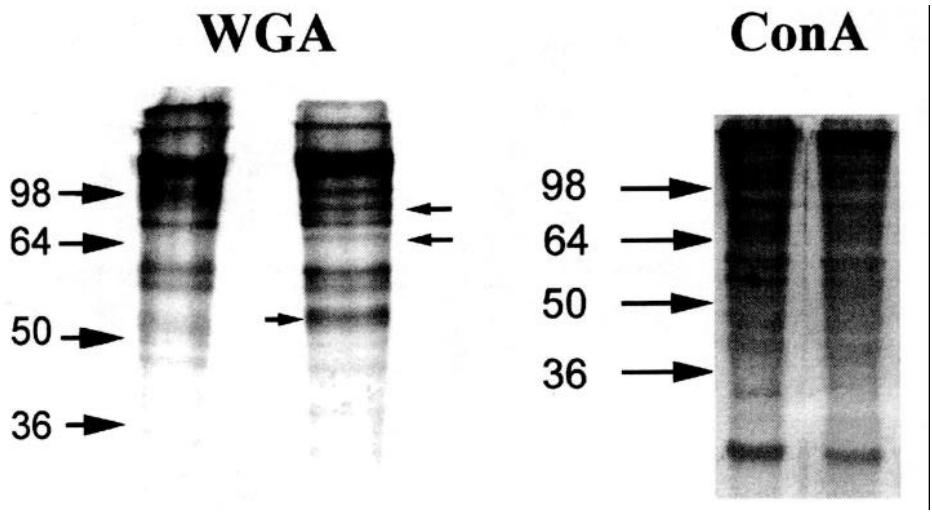
Figure 1. Normal gastrula at 36 hr with extended gut (arrows) and ventrolateral mesenchyme cells (arrowheads). **Figure 2.** MXC-exposed gastrula lacking a well defined gut and with scattered mesenchyme cells (arrowheads) in the blastocoel.

determined by Lowry assay (Lowry *et al.* 1951), were loaded and electrophoresed (Laemmli 1970) using a Bio-Rad Mini-Protean II system

Prestained molecular weight standards (Sigma) were also included. After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) using a Bio-Rad Trans blot electrophoretic transfer cell. The membranes were incubated in TTBS for 30 min and pre-quenched with 3% H_2O_2 for 10 min to eliminate any endogenous peroxidase activity (Graham *et al.* 1995). They were then incubated in 10 µg/mL solution of biotinylated lectin (Con-A or WGA; Vector Labs, Inc.) in TTBS for 1 hr. After 3 washes with TTBS, the membranes were incubated at RT for 1 hr with avidin-horseradish peroxidase complex (Vector Labs). After 3 washes with TTBS, the blots were incubated with H_2O_2-diaminobenzidine plus nickel chloride (Vector Labs) until suitable color intensity was obtained. The blots were washed with 2 changes of distilled water over 10 min, allowed to air dry and then photographed.

RESULTS AND DISCUSSION

Acute MXC-exposure of zygotes at 10 min PI resulted in abnormal morphogenesis and development. A few embryos were arrested in development before hatched blastula (22 hr) and eventually degenerated. At mesenchyme blastula, most of the MXC-exposed embryos had thickened vegetal plate epithelium and loose mesenchyme cells scattered within the blastocoel showing no discernible pattern although the individual cells appeared normal. At gastrula stage (36 hr), control embryos had normal guts that extended across the blastocoel with primary mesenchyme cells clustered in the ventrolateral aspect of the blastocoel on either side of the invaginating gut (Fig. 1). The majority of MXC-exposed embryos had rudimentary (abnormally short) guts or no guts at all. These embryos had many mesenchyrne cells clustered at the vegetal pole or dispersed throughout the blastocoel (Fig. 2).



3. DMSO MXC **4.** DMSO MXC

Figure 3. Western blot of homogenized 36 hr gastrulae stained with biotinylated WGA. Note the appearance of three unique bands at approximately 54, 64, and 90 kDa (small arrows) in the lane of MXC-treated gastrulae. Figure 4. Western blot of homogenized 36 hr gastrulae stained with biotinylated Con-A. No new bands are observed in the lane of MXC-treated gastrulae. The large arrows indicate the positions of molecular weight markers.

In order to ascertain whether the above-described morphological changes were correlated with changes in glycoprotein composition, Western blots of those early gastrula-stage embryos were probed with biotinylated WGA or biotinylated Con-A. Homogenates of the MXC-exposed embryos show the appearance of three new bands with affinity for WGA (54 kDa, 64 kDa and 90 kDa). These bands do not appear in the controls (Fig. 3). On the other hand, Western blots of early gastrulae probed with Con-A show that there was no qualitative difference in the pattern of total Con-A-binding glycoproteins between MXC-exposed embryos and the controls (Fig. 4).

Acute MXC-exposure of sea urchin embryos (10 min PI) resulted in abnormal gastrulation. The time of onset and the nature of the abnormalities suggested that MXC interfered at some level with either intercellular interactions or altered the molecular cues of the extracellular matrix (ECM). Therefore, labeled lectins were used to probe the expression of glycoproteins during gastrulation. Gastrulation is normally preceded by an increased synthesis of glycoproteins and extracellular glycosaminoglycans within the blastocoel (Lennarz 1983). This is accompanied by a concomitant development of blastocoelic ECM composed of fibrillar sheets interconnected by crossbridges (Cherr *et al.* 1992).

During the initial phase of sea urchin gastrulation, a number of changes take place including changes in the distribution of lectin-binding sites. Con-A, whose specific binding sugar moiety is mannose, has been shown to bind to the basal laminae of the animal half of developing embryos and the base of the archenteron (Katow and Solursh 1982; DeSimone and Spiegel 1986a), WGA, whose specific hapten sugar is N-acetyl-β-glucosamine, has been shown to bind exclusively to primary mesenchyme cells (Spiegel and Burger 1982). Recently, another carbohydrate-containing determinant was identified that is concentrated in the vegetal basal laminae and ventral aspects of the embryo (Ingersoll and Ettensohn 1994). The migration of mesenchyme cells during gastrulation also uses the blastocoelic matrix as a substratum for attachment and cell guidance (Ettensohn and McClay 1986). Alteration or reduction of the guidance cues within the blastocoel by MXC may explain why mesenchymal cells were scattered throughout the blastocoel of MXC-exposed embryos resulting in stunted guts.

Examination of Western blots of gastrulae probed with WGA showed that there were three new (extra) bands (54 kDa,, 64 kDa and 90 kDa) in MXC-exposed embryos compared to controls. This suggests that MXC may have unmasked or induced an abnormal synthesis of WGA-binding material within the gastrulae. However, the possibility that MXC might have prevented degradation of these three proteins from earlier embryonic stages cannot be discounted at this time. Sea urchin embryos have been shown to express unique high molecular weight glycoproteins in response to exposure to petroleum waste (Baldwin et al. 1992). Changes in the glycoprotein composition within the gastrulae may reflect alterations of the molecular microenvironment within the blastocoel. This may, disrupt the normal ECM-dependent cellular processes necessary for normal gastrulation and spiculogenesis (Burke and Tamboline 1990). The biochemistry of the newly appearing glycoproteins and the actual mechanism of MXC perturbation remain to be investigated.

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