The effects of ethanol on embryonic actin: A possible role in teratogenesis

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Summary. When the neural crest is cultured in the long or short term presence of ethanol, monoclonal anti-actin reveals the development of a disorganized actin cytoskeleton. In the long term, many cells fail to differentiate morphologically, whereas in the short term already differentiated cells rapidly alter their shape and their cell-to-cell contacts.
Key words. Ethanol; neural crest cells; teratogenesis; actin.

Ethanol is a known teratogen and causes fetal alcohol syndrome (FAS)[1]. Tissue anomalies manifested in FAS have recently been related to dysfunction in neural crest migration and histogenetic patterning[2,3], both of which are actin-dependent processes[4,5]. In vitro, neural crest cells migrate away from the neural tube explant over the course of several days. Usually by the end of the third day the migratory phase ends, and cells begin to differentiate morphologically. We show here for the first time, using fluorescence with monoclonal anti-actin, that over a wide range of commonly observed human blood levels (0.05–0.20%), ethanol disrupts the organization of the neural crest cell actin cytoskeleton. Though the treated cells are able to migrate in the continuous presence of ethanol, many are unable to differentiate completely into their characteristic, dendritic morphology. Short-term ethanol treatment of well differentiated cells rapidly induces abnormalities in the actin cytoskeleton accompanied by changes in cell shape and cell-to-cell contacts. Because actin provides motive forces during histogenesis, we reason that ethanol-induced changes in the actin organization of neural crest cells may be implicated in the etiology of morphogenetic anomalies seen in the fetal alcohol syndrome.

The neural crest is a transient embryonic tissue which forms dorsal to the neural tube. It is subdivided into cranial and trunk regions. While these two segments provide a variety of phenotypes, both give rise to mesenchyme and pigment cells. A correlation exists between clinically recognized malformations and the known morphogenetic fates of neural crest mesenchyme[2,3]. These mesenchymal cells assist in forming the face, lower jaw, tongue, thyroid and parathyroid[3], all, or some of which, may be malformed in FAS.

Ethanol has been shown to interfere with various morphogenetic processes, including glial and neuronal cell migration[6], neurite elongation[7], axonal distribution[8], dendritic branching[9], and the shape of fusing neural fold cells[10]. Because actin, in association with myosin[11], provides motive forces for cell migration and for morphological differentiation, we reasoned that ethanol-induced defects of neural crest derivatives may be linked to insult at the level of the actin cytoskeleton. To test this possiblity, urodele neural crest cells were cultured, either in the continuous presence of ethanol for six days or with a single, 2 h 20 min exposure to ethanol, only on day 6. Monoclonal antibodies were employed against the actin of ethanol-treated and control cells and studied microscopically.

Cultures were grown from explants of trunk and posterior cranial neural tube and crest removed from embryos of the salamander *Ambystoma maculatum* at stages 21 through 26 (Harrison)[12]. In all, 51 explants were cultured in Niu-Twitty's complex salt solution, pH 7.4. In one series, cultures were exposed continuously to ethanol for six days (0.05%, 0.10%, 0.15%, or 0.20%). Solutions were made fresh and changed every 24 h. In another series, cultures were exposed to ethanol (0.10%, 0.15% or 0.20%) for a period of 2 h 20 min only on day 6. After this short-term exposure, several cultures were removed from ethanol and cultured in ethanol-free culture medium for an additional 22 h to determine whether affected cells can recover. We assessed the ability of ethanol-affected cells to synthesize both DNA and protein by labeling with tritiated (^{3}H) thymidine (18.3 Ci/mM) and leucine (19.7 Ci/mM), Amersham, Arlington Hts., Ill. Cells were first exposed to ethanol (0.2%) for 45 min during which time many exhibited retraction. The cells were then exposed to ethanol for an additional 30 min in the presence of a 100 µCi of either ^{3}H thymidine or leucine. The cultures were washed 5 times in Niu-Twitty's solution. All fixation was done in 3.7% formaldehyde in Niu-Twitty's solution for 1 h. Autoradiographs were prepared by coating the slides containing the cultures with Kodak AR-100 stripping film and incubating for 4 or 5 weeks at 4 °C and developed in Kodak D-19 for 6 min.

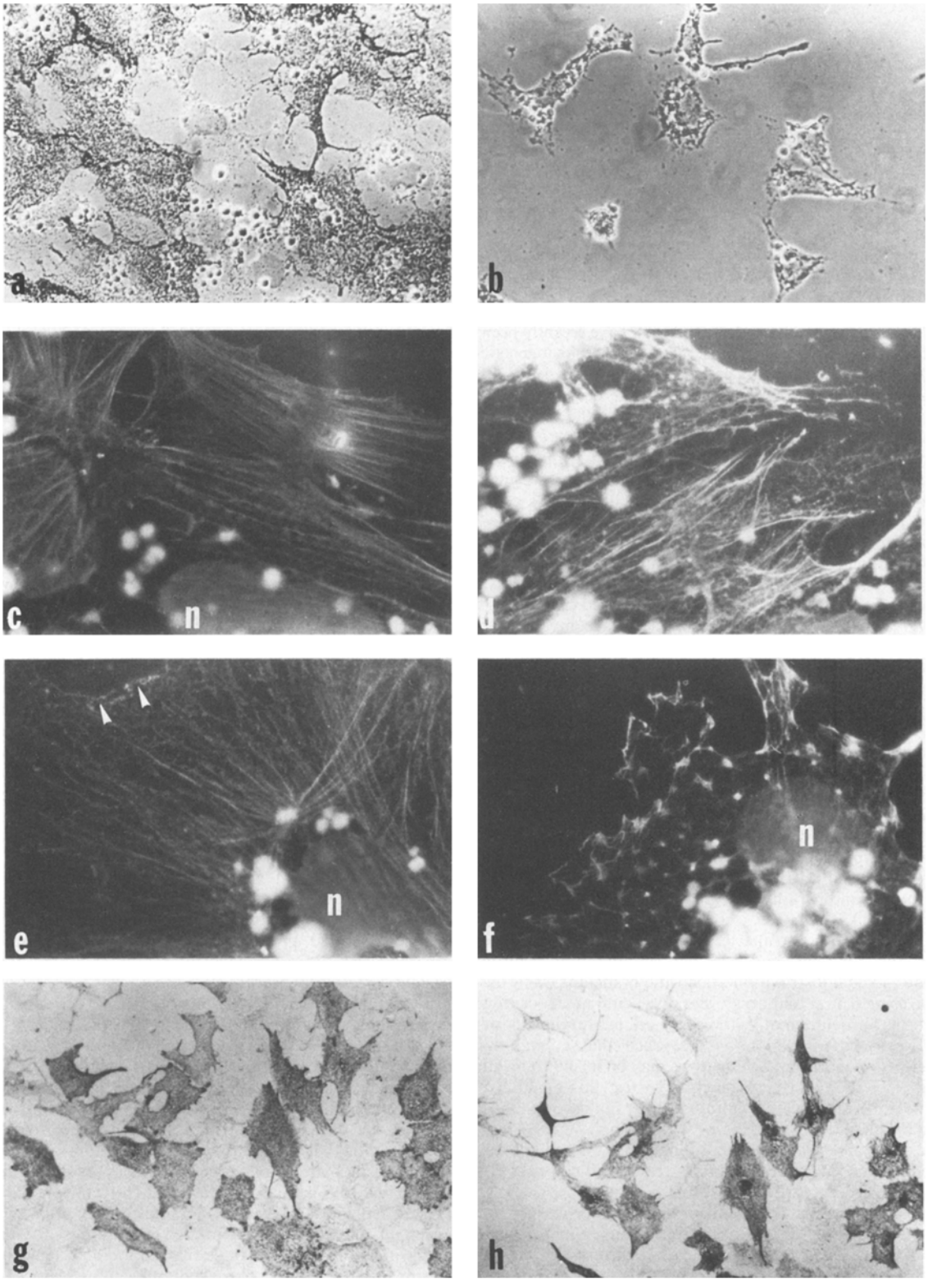
After fixation the cells were washed three times in phosphate buffered saline (PBS) and made permeable to monoclonal antibodies by immersion in acetone at −20 °C for 15 s. After washing in phosphate buffer, cells were exposed to mouse monoclonal anti-actin antibodies (Amersham, Arlington Hts., Ill.) at 37 °C for 1 h. The cultures were again washed three times in PBS and treated with fluorescein-labeled sheep anti-mouse Ig (Amersham) for 1 h at 37 °C. To remove unbound Ig, the cells were washed three times in PBS and dehydrated in ascending concentrations of ethanol to xylene. Coverslips were mounted with Eukitt nonfluorescence medium (Calibrated Instruments, Ardsley, N.Y.). Observations were made with a Zeiss standard microscope equipped with phase, Nomarski, and fluorescence optics, the latter with filters system BP 485, FT 510, LP 520. Cultures were photographed with a Zeiss MC 63 camera system using Kodak Tri-X film.

In control cultures, after six days, trunk and posterior cranial neural crest cells have migrated away from the neural tube explant and form a monolayer outgrowth composed mainly of mesenchyme and pigment cells (fig. a). As differentiation proceeds, cells acquire a dendritic morphology that gradually becomes extensively branched.

With continuous ethanol treatment (0.05, 0.10, 0.15 or 0.20%) for six days, cultures remained viable and cells migrated away from the neural tube as they do in the controls. However, in each of the above concentrations, many cells do not complete morphological differentiation. Such cells are contracted (fig. b) and do not exhibit the normal, extensively branched dendritic morphology. No differing effects are visible with increasing ethanol concentrations (0.05–0.20%). In control cultures there are two characteristic patterns of the actin network, as revealed by the monoclonal anti-actin + Ig complex. Most control cells exhibit either a reticular arrangement or parallel array of linear fibers, which surround the centrally-located nucleus (fig. e). With phase optics it can be seen that these filaments end at, or within, the plasmalemma. In ethanol-treated cells, fluorescence labeling reveals that the linear actin fibers are matted and thickened (fig. d).

Some control cells present a somewhat different actin pattern (fig. e); the linear bundles of actin, instead of terminating at the membrane, lead into a fine filigree. This filigree is especially evident along the membrane fringe (arrows in fig. e). In ethanol-treated cells the actin filigree near the cell membrane becomes thickened and clumped (fig. f).

Short-term administration (2 h 20 min) of ethanol (0.10, 0.15, or



Control cells are in the left column; ethanol-treated cells in the right column. a) Phase micrograph of a control day-6 culture showing arborized, dendritic mesenchyme cells and melanocytes. × 500. b) Phase micrograph of a day-6 culture treated continuously from explantation with 0.20% ethanol. Cells migrate but exhibit limited morphological differentiation. The dendritic processes of affected cells are shorter and have fewer branches. × 500. c) Fluorescence micrograph of a day-6 control culture illustrating the nucleus (N) and evenly tapered fibers that stain with monoclonal anti-actin. The refractile bodies are yolk platelets. × 2250. d) Fluorescence micrograph of a day-6 ethanol-treated (continuous exposure) culture illustrating a comparable region to that shown in figure c. Here, the actin cytoskeleton is thickened and disrupted. × 2250. d) Fluorescence micrograph of a day-6 culture illustrating the nucleus (N) and evenly tapered linear actin fibers that extend into a fine filigree along the plasma membrane (arrows). × 2250. f) Fluorescence micrograph of a day-6 ethanol-treated (continuous exposure) culture illustrating a comparable region to that shown in figure e. Here, the linear actin fibers are disrupted and extend into thickened or irregular clumps. × 2250. g) Bright-field micrograph of a living day-6 control culture prior to the administration of 0.2% ethanol. Both mesenchyme cells and melanocytes exhibit dendritic arborization. × 170. h) Bright-field micrograph of the same cells shown in figure g after treatment with 0.2% ethanol for 2 h 20 min. Cell margins are retracted and cell-to-cell contacts are altered. × 170.

0.20%) to day-6 cultures induces progressive retraction of the cell margin. Figures g and h show the same living cells before (fig. g) and after (fig. h) ethanol treatment). In addition to inducing the retraction of cell processes, the single exposure to ethanol alters existing cell-to-cell contacts. When cells are removed from ethanol for 22 h, effected cells remain unable to develop normal processes whereas uneffected cells in the same cultures continue to differentiate. Moreover, autoradiographs reveal that ethanol-effected cells (2 h 20 min) remain capable of incorporating tritiated thymidine or leucine and synthesize them into DNA and protein respectively. Tritiated thymidine is exclusively incorporated above the nuclei whereas the leucine label is found over the entire cell. The fluorescent actin pattern following short-term ethanol treatment is similar to that observed following long-term ethanol treatment (figs d and f). Using Nomarski optics, we determined that ethanol-induced changes are not accompanied by cell lysis.

Cell shape changes are triggered by ethanol in a number of systems[6–10], and this uniform cellular response underscores the likelihood that actin is a teratogenic target. In particular, ethanol induces changes in the dendritic arborization of neurons in the cerebral cortex[9] and neurite elongation of chick embryo sensory and spinal cord neurons[7]. Our findings corroborate and extend these studies. After ethanol treatment, many neural crest cells do not undergo dendritic branching, a condition we show to be correlated with a disorganized actin cytoskeleton. These effects in our culture system are due to ethanol alone because there is no enzymatic conversion to acetaldehyde or acetate in isolated neural tubes and crest cells. However, it is important to stress that in situ the ethanol byproducts acetaldehyde and acetate may pass from the maternal to the fetal circulation and therefore possibly play a role in morphogenetic alteration[1].

The known ability of cells to develop a metabolic and functional tolerance to continuous ethanol exposure may account for the presence of some cells with a normal morphology[1]. At present, we do not know whether ethanol's effect upon actin is direct or indirect. Consideration of ethanol's insult is made difficult by actin's intimate association with the cell membrane, which is known to become more fluid in the presence of ethanol[13]. Specifically, the ethanol permeation may affect a reconfiguration of proteins and lipoproteins of the plasmalemma[14]. Membrane proteins play an important role as select linkers of the actin cytoskeleton to the membrane and probably participate in the restructuring of the cytoplasmic matrix during morphological differentiation[15]. Also, an ethanol-induced change in membrane proteins and lipoproteins may induce altered ionic fluxes[14]. There is evidence from brain studies that ethanol reduces membrane-bound Ca^{2+}[16]. This reaction to ethanol could have important morphogenetic consequences because the Ca^{2+} pool, as well as its related fluxes, controls actin-assisted contraction and relaxation. Moreover, it is possible that the functional association of actin microfilaments (5–8 nM) and microtubules (tubulin, 25 nM) is somehow affected[17].

Morphogenetic dysfunction of the neural crest in situ could conceivably be expressed at the level of cell migration and/or morphological differentiation[3]. While in situ, ethanol may cause subtle alterations in the migratory pattern of neural crest cells, it is clear from the present study that even after continuous treatment, neural crest cells remain capable of migrating in vitro and that these cells maintain their ability to synthesize both DNA and protein. What is shown for the first time in this study is that ethanol treatment markedly disrupts the organization of the actin cytoskeleton and prevents normal dendritic branching in many cells. Even when applied in the short-term to well-differentiated neural crest cells, ethanol rapidly disrupts the actin pattern, promotes cell retraction, and alters cell-to-cell filopodial contacts.

During morphogenesis, cellular interactions are dependent upon precise contact at cellular interfaces. We suggest, therefore, that ethanol-induced malformations may result from the inability of aberrently formed neural crest cells (i.e., mesenchyme) to interact normally with the primordial tissues that form craniofacial structures. Our findings may provide a basis for further investigations on how ethanol causes the morphogenetic anomalies manifested in FAS.

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Differential expression and dosage compensation of the α-amylase gene in *Drosphila miranda*[1]

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Summary. The α-amylase gene of *Drosophila miranda* is located on the X^2- and on the neo-Y-chromosome, both developing sex chromosomes. Crosses between strains carrying different electrophoretically distinguishable alleles of the α-amylase gene were performed. Females of the F_1 offspring showed the expected heterozygosity, while the males proved to be hemizygous for this locus. Only the gene on the X^2-chromosome is expressed, whereas the corresponding gene on the neo-Y-chromosome is not. Estimates of the α-amylase activity in crude homogenates of male and female flies suggest strongly that the α-amylase gene is dosage compensated in *D. miranda*. In contrast to this situation, in all other *Drosophila* species the α-amylase allele is autosomal and hence not dosage compensated.

Key words. Differential gene expression; *Drosophila miranda;* dosage compensation; α-amylase.