Int. J. Dev. Biol. 43: 167-174 (1999)

Original Article

Alcohol promotes *in vitro* chondrogenesis in embryonic facial mesenchyme

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ABSTRACT Ethanol is a well-recognized teratogen in vertebrates that can perturb the development of the facial primordia and various other embryonic structures. However, the mechanisms underlying alcohol's effects on embryogenesis are currently unclear. Recent evidence suggests that the cranial neural crest, which forms the entire facial skeleton, may be a particularly sensitive target of ethanol teratogenicity. In the present study we have examined the influence of *in vitro* ethanol exposure on cartilage differentiation in micromass cultures of mesenchymal cells isolated from the various facial primordia (maxillary, mandibular, frontonasal, and hyoid processes) of the stage 24 chick embryo. In all four populations of facial mesenchyme, exposure to 1-1.5% ethanol promoted marked increases in Alcian blue-positive cartilage matrix formation, a rise in $^{35}SO_4$ accumulation into matrix glycosaminoglycans, and enhanced expression of cartilage-characteristic type II collagen and aggrecan gene transcripts. In frontonasal and mandibular mesenchyme cultures, which undergo extensive spontaneous cartilage formation, ethanol treatment quantitatively elevated both matrix production and cartilage-specific gene transcript expression. In cultures of maxillary process and hyoid arch mesenchyme, which form little or no cartilage spontaneously, ethanol exposure induced the formation of chondrogenic cell aggregates and the appearance of aggrecan and type II collagen mRNAs. These actions were not restricted to ethanol, since tertiary butanol treatment also enhanced cartilage differentiation in facial mesenchyme cultures. Our findings demonstrate a potent stimulatory effect of alcohol on the differentiation of prechondrogenic mesenchyme of the facial primordia. Further analysis of this phenomenon might yield insight into the developmental mechanisms underlying the facial dysmorphologies associated with embryonic ethanol exposure.

KEY WORDS: *alcohol, ethanol, cartilage, facial chondrogenesis*

Introduction

The vertebrate facial skeleton (including the frontal bone, nasal septum, maxilla, hard palate, mandible, and hyoid apparatus) differentiates from neural crest-derived ectomesenchyme cells that invade the embryonic frontonasal, maxillary, mandibular, and hyoid prominences (Noden, 1983; Couly *et al.*, 1993). The relative extents of osteogenic versus chondrogenic differentiation in these facial primordia differ considerably, forming cartilaginous structures at some sites (e.g., Meckel's cartilage of the mandible) and undergoing direct intramembranous ossification at other locations (e.g., the maxillary processes and their palatal derivatives). Mesenchymal cells from the four embryonic facial primordia also display markedly different tendencies for spontaneous chondrogenic differentiation when cultured *in vitro* (Wedden *et al.*, 1986; Kulyk and Reichert, 1992). Frontonasal and mandibular mesenchymes undergo extensive cartilage formation in high-density "micromass" cultures, whereas mesenchymal cells from the maxillary and hyoid arches will spontaneously form little or no cartilage *in vitro*. The different behaviors of these facial mesenchyme populations *in vitro* reflect, in part, their determinations for distinct skeletogenic fates *in situ*, since transplantation studies indicate that facial neural crest populations are at least partially committed to form specific skeletal structures even before emerging from their sites of origin in the cephalic neural folds (Noden, 1983, 1988). In

Abbreviations used in this paper: ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; DMEM, Dulbecco's Modified Eagle's Medium; EtOH, ethanol; FAS, Fetal Alcohol Syndrome; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GAG, glycosaminoglycan; *t*-butanol, tertiary butanol.

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0214-6282/99/$10.00

Printed in Spain
www.lg.ehu.es/ijdb

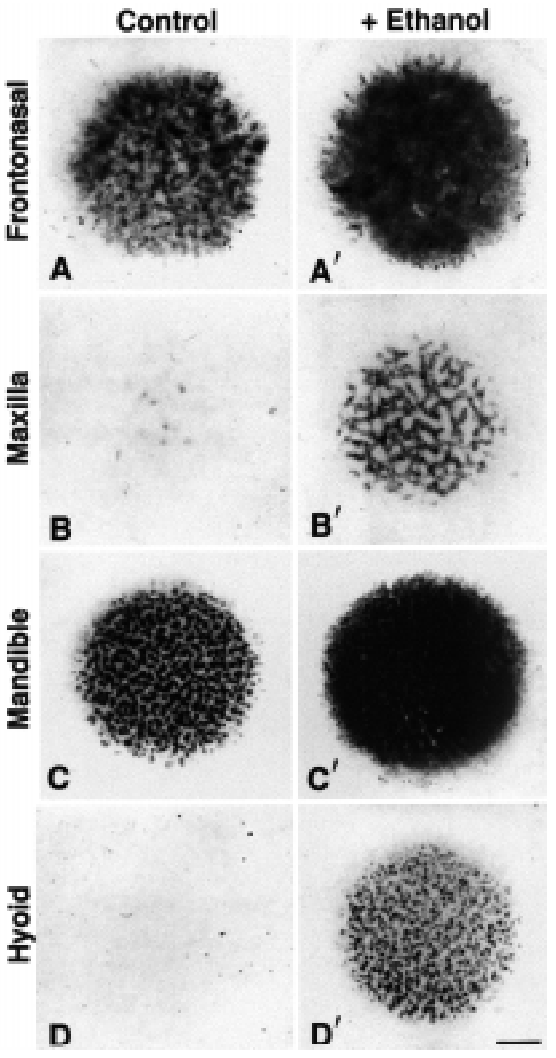

Fig. 1. Micromass cultures of stage 24 chick embryo facial mesenchyme cells that were incubated for 3 days in the absence (control) or presence of 1.0-1.5% ethanol, then stained with Alcian blue to demonstrate the distribution of cartilage matrix. *Cultures of both frontonasal process* **(A)** *and mandibular process* **(C)** *mesenchymes spontaneously developed numerous Alcian blue-positive chondrogenic cell aggregates. Continuous exposure to 1.0% ethanol markedly elevated cartilage matrix production in the frontonasal* **(A')** *and mandibular* **(C')** *mesenchyme cultures, yielding sheets of cartilage tissue that stained intensely with Alcian blue. Control cultures of maxillary* **(B)** *and hyoid process* **(D)** *mesenchymes formed little or no Alcian blue-stainable cartilage matrix. In contrast, the presence of 1.5% ethanol induced the differentiation of numerous Alcian blue-positive chondrogenic cell aggregates in the maxillary and hyoid mesenchyme cultures* **(B',D').** *Bar, 1 mm for all photographs.*

addition, differences in the proportions of mesoderm-derived myogenic cells versus neural crest-derived skeletogenic cells that populate the individual facial primordia can affect their relative capacities for *in vitro* cartilage formation (Ralphs *et al.*, 1989; Kulyk and Reichert, 1992).

Ethanol is a well-documented teratogen in vertebrates, which can induce a wide spectrum of malformations in embryonic organ primordia, including skeletal, cardiac, central nervous system, and urogenital structures (Herrmann *et al.*, 1980; Streissguth *et al.*, 1980; Webster *et al.*, 1983; Becker *et al.*, 1996). Facial malformations, in particular, are a diagnostic feature of Fetal Alcohol Syndrome (FAS) in humans (Johnson *et al.*, 1996) and are among the most common defects induced by gestational alcohol exposure in laboratory animals (Sulik *et al.*, 1981; Webster and Ritchie, 1991). The embryopathic effects of alcohol have been traditionally ascribed to generalized, systemic disturbances of nutrient uptake, essential metabolism, cellular proliferation, and cell viability (Schenker *et al.*, 1990; Shibley and Pennington, 1997). However, recent investigations indicate that ethanol may also exert more selective influences on specific progenitor cell populations of embryonic organ primordia. In particular, mounting evidence suggests that the cranial neural crest is a particularly sensitive target of ethanol teratogenicity (Cartwright and Smith, 1995a,b; Rovasio and Battiato, 1995; Chen and Sulik, 1996). Recently we reported that ethanol exposure enhances both cartilage matrix production and cartilage-specific gene expression in cultures of embryonic chick limb mesenchyme cells, suggesting the possibility that ethanol might exert a selective action on the differentiation of skeletogenic progenitor cells (Kulyk and Hoffman, 1996). In the present study we have extended our analysis to examine the effects of ethanol on cartilage differentiation in cultures of mesenchymal cells isolated from the various facial primordia (frontonasal, maxillary, mandibular, and hyoid processes) of the stage 24 chick embryo. We demonstrate that ethanol exposure quantitatively elevates cartilage matrix deposition and levels of both type II collagen and aggrecan transcripts in cultures of spontaneously chondrogenic frontonasal and mandibular process mesenchymes. Moreover, ethanol treatment induces chondrocyte-specific gene expression and cartilage matrix deposition even in cultures of maxillary and hyoid process mesenchymes, which undergo little or no cartilage differentiation spontaneously. These findings suggest the possibility that embryonic alcohol exposure might perturb facial morphogenesis through a relatively direct action on the differentiation of neural crest-derived chondrogenic progenitor cells of the facial primordia.

Results

Micromass cultures of facial mesenchyme cells isolated from the frontonasal, maxillary, mandibular and hyoid processes of the stage 24 chick embryo display qualitatively distinct patterns of spontaneous chondrogenic differentiation during a 3 day period of incubation (Wedden *et al.*, 1986; Kulyk and Reichert, 1992). Mesenchyme cells isolated from the frontonasal and mandibular processes exhibit relatively high levels of spontaneous cartilage differentiation when cultured in control medium. Specifically, the frontonasal process mesenchyme forms irregularly shaped patches or sheets of differentiating chondrocytes that elaborate an Alcian blue-stainable matrix containing sulfated glycosaminoglycans (GAG) (see Fig. 1A). Mandibular process mesenchyme develops numerous spheroidal, Alcian blue-positive nodules of chondrogenically differentiating cells which are separated by nonchondrogenic tissue (Fig. 1C). In contrast, mesenchymal cells from the maxillary processes and hyoid arches elaborate little or no Alcian blue-positive cartilage matrix during a 3 day period of culture in control medium (Fig. 1B,D).

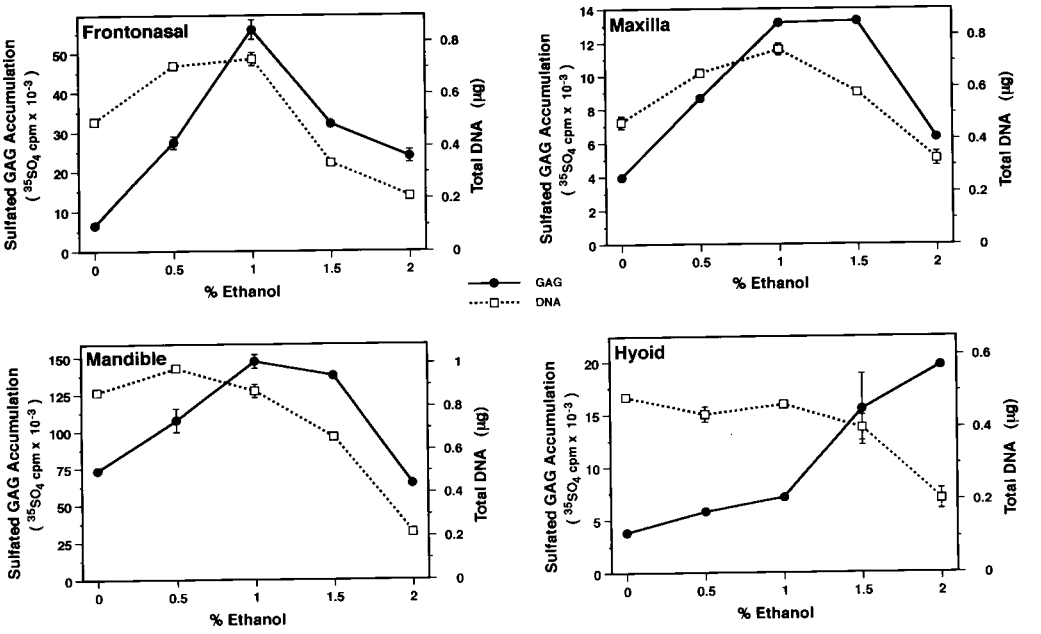

Fig. 2. Graphs illustrating the effects of various concentrations of ethanol on the incorporation of $^{35}SO_4$ label into total sulfated GAG (sum of cell layer- and medium-associated GAG; closed symbols) as well on total DNA accumulation (open symbols) during a 3 day period of incubation in micromass cultures prepared from mesenchymal cells of the frontonasal, maxillary, mandibular and hyoid processes of the stage 24 chick embryo. *Data points represent the mean ± standard error of determinations from four replicate cultures. (Error bars that are not visible lie within the dimensions of the data point illustrated).*

Our initial experiments examined the effects of various ethanol concentrations on the incorporation of $^{35}SO_4$ label into sulfated GAG (a quantitative index of cartilage matrix proteoglycan production) and on total DNA accumulation (indicative of cell number) in micromass cultures prepared from mesenchyme cells of the frontonasal, maxillary, mandibular and hyoid primordia. In all four facial mesenchyme populations, the presence of ethanol in the culture medium promoted a striking dose-dependent increase in total sulfated GAG accumulation relative to parallel cultures incubated in control medium (Fig. 2). In frontonasal, maxillary, and mandibular mesenchyme cultures, statistically significant elevations of sulfated GAG accumulation were observed with as little as 0.5% (v/v) ethanol ($P<0.01$; ANOVA, Dunnet's multiple comparisons test). Maximum stimulation of GAG production was observed at doses of 1% ethanol in cultures of frontonasal and mandibular mesenchyme (8.6- and 2.0-fold higher than control cultures, respectively) and at 1.5% ethanol in maxillary mesenchyme (3.4-fold increase). In cultures of hyoid mesenchyme, concentrations of 1.5-2.0% ethanol promoted over 4-fold increases in sulfated GAG production (significant at $P<0.05$).

In addition, ethanol had variable effects on DNA accumulation in the facial mesenchyme cultures. There was a notable reduction in total DNA accumulation relative to control cultures in frontonasal and mandibular mesenchyme treated with 1.5-2.0% ethanol, and in maxillary and hyoid mesenchyme exposed to 2.0% ethanol (Fig. 2). Lower concentrations of ethanol moderately increased the DNA content of frontonasal, and maxillary cultures, and slightly decreased DNA accumulation in hyoid mesenchyme cultures (Fig. 2). We employed concentrations of 1.0% ethanol (for mandibular and frontonasal cultures) and 1.5% ethanol (for maxillary and hyoid cultures) in all subsequent experiments since these doses stimulated maximal or near-maximal increases in GAG production (2- to 8-fold increases) while only modestly affecting total DNA accumulation (0.8 X, 1.0 X, 1.3 X and 1.5 X control levels for hyoid, mandibular, maxillary, and frontonasal cultures, respectively). In this manner, the influence of ethanol on chondrocyte differentiation could be preferentially examined while minimizing treatment effects on cell number and density.

Histochemical staining with Alcian blue confirmed that ethanol treatment induced striking elevations in matrix proteoglycan deposition in cultures of mesenchymal cells from all four facial primordia (Fig. 1A',B',C',D') when compared to cultures maintained in control medium (Fig. 1A,B,C,D). In frontonasal and mandibular process mesenchymes, which are spontaneously chondrogenic *in vitro* (Fig. 1A,C), exposure to 1.0% ethanol promoted quantitative increases in cartilage matrix accumulation such that the cultures formed nearly continuous sheets of cartilage tissue that stained intensely with Alcian blue (Fig. 1A',C'). In cultures of maxillary and hyoid arch mesenchyme, which elaborated little or no histochemically identifiable cartilage matrix when maintained in control medium (Fig 1B,D), the presence of 1.5% ethanol induced the formation of numerous Alcian blue-positive aggregates of chondrogenically differentiating cells (Fig. 1B',D'). The stimulatory effect of the alcohol on matrix GAG accumulation was not simply due to a shift in the ratio of cell layer-associated versus medium-associated GAG deposition. As shown in Figure 3, ethanol treatment elevated $^{35}SO_4$ accumulation into pericellular GAG and medium-secreted GAG to similar extents.

Ethanol exposure also enhanced the expression of mRNA transcripts for type II collagen and aggrecan, the major constituents of cartilage matrix. Type II collagen and aggrecan mRNAs were undetectable in hyoid arch mesenchyme after 3 days of culture in control medium, whereas transcripts for both cartilage-characteristic genes were abundant in hyoid cultures incubated in the presence 1.5% ethanol (Fig. 4). In cultures of maxillary process mesenchyme, 1.5% ethanol treatment promoted a 5.6-fold increase in type II collagen mRNA levels (average of two independent experiments) and induced the expression of aggrecan transcripts, which were nearly undetectable in parallel control cultures (Fig. 4). In cultures of the spontaneously chondrogenic frontonasal process mesenchyme, 1% ethanol treatment promoted increases of 3.4- and 3.8-fold in the steady-state levels of type II collagen and

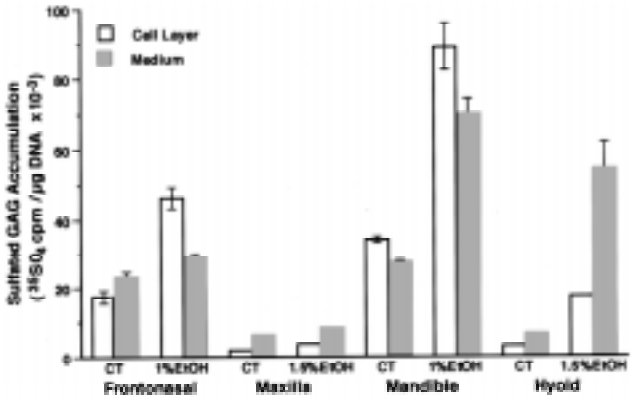

Fig. 3. Histograms illustrating levels of $^{35}SO_4$-labeled GAG deposited in the cell layer and secreted into the culture medium of 3-day micromass cultures of chick embryo facial mesenchyme cells incubated in the presence of 1.0-1.5 % ethanol (EtOH) or in control medium (CT) lacking ethanol. *GAG accumulation is expressed per μg total cellular DNA to correct for modest differences in the DNA content of control and ethanol-treated cultures. Error bars represent the mean ± standard deviation of determinations from four replicate cultures. (Error bars that are not visible lie within the dimensions of the data point illustrated). Note that ethanol treatment promoted elevations in both cell layer- and medium-associated GAG accumulation.*

aggrecan transcripts, respectively (means of two independent experiments). Even in cultures of mandibular process mesenchyme, which exhibited the highest control levels of type II collagen and aggrecan gene expression (Fig. 4), treatment with 1% ethanol raised type II collagen and aggrecan mRNA expression by averages of 1.6- and 2.1-fold, respectively.

The stimulatory effects of alcohol on *in vitro* facial cartilage differentiation were not restricted to ethanol, since treatment with *t*-butanol caused a similar increase in chondrogenic differentiation in the facial mesenchyme cultures (Fig. 5). Stimulation of chondrogenesis was optimal at 0.5% *t*-butanol (v/v) in frontonasal and mandibular mesenchyme cell cultures and at 1% *t*-butanol in maxillary and hyoid mesenchyme cultures (dose-response data not shown). At these concentrations, *t*-butanol treatment stimulated significant increases in both cell layer- and medium-associated GAG accumulation within the frontonasal, maxillary, mandibular, and hyoid mesenchyme cultures (Fig. 5). In addition, *t*-butanol treatment promoted striking increases in Alcian blue-positive matrix accumulation resembling those induced by ethanol treatment (not shown).

Discussion

We have demonstrated that ethanol exposure has a striking stimulatory effect on chondrogenic differentiation in cultures of mesenchymal cells isolated from the facial primordia of the chicken embryo. In micromass cultures of frontonasal and mandibular process mesenchyme, which undergo extensive spontaneous chondrogenesis, ethanol treatment promoted quantitative increases in Alcian blue-positive cartilage matrix accumulation and sulfated GAG deposition. Moreover, in maxillary and hyoid mesenchyme cultures, which form little or no cartilage spontaneously, ethanol exposure induced the formation of Alcian blue-positive chondrogenic cell aggregates. RNA analysis demonstrated that alcohol exposure enhanced the expression of transcripts for both type II collagen and aggrecan, the principal cartilage matrix components, confirming that it acts at a pre-translational level in elevating cartilage matrix production by the facial mesenchyme cells.

Another aliphatic alcohol, *t*-butanol, provoked a similar enhancement of cartilage differentiation in the facial mesenchyme cultures. Unlike ethanol, *t*-butanol is not metabolized to acetaldehyde (Williams, 1959; Wood and Laverty, 1979), a product which may itself have teratogenic actions (Webster *et al.*, 1983). Thus the stimulatory effects of ethanol and *t*-butanol on facial chondrogenesis appear to be properties of the alcohols themselves rather than a downstream metabolite. Indeed, we've previously shown that methanol and propanol are also effective stimulants of *in vitro* cartilage differentiation in cultures of embryonic chick limb bud mesenchyme (Kulyk and Hoffman, 1996).

Our facial mesenchyme cultures contain a mixed population of mesoderm-derived myogenic progenitor cells and neural crest-derived ectomesenchyme cells. Since the latter form all cartilage, bone, and connective tissues of the face *in vivo* (Noden, 1983), they are most likely the cells responsive to ethanol's cartilage-inducing effects. Indeed, we have previously shown that ethanol stimulates cartilage differentiation in cultures prepared from distal

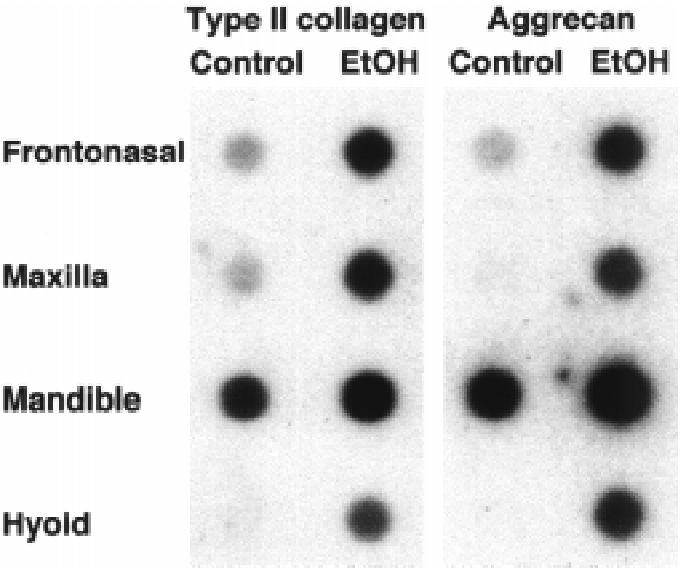

Fig. 4. Autoradiographs representing steady-state levels of type II collagen and aggrecan transcripts in total RNA isolated from 3-day micromass cultures of frontonasal, maxillary, mandibular and hyoid process mesenchyme maintained in the absence (controls) or presence of 1-1.5% ethanol. *Maxillary and hyoid cultures were treated with 1.5% ethanol; frontonasal and mandibular mesenchymes were treated with 1.0% ethanol. (The signals for mandibular cultures in the autoradiograph shown are over-exposed due to the greater abundance of aggrecan and type II collagen mRNAs in mandibular cultures relative to other facial mesenchymes. However densitometric measurements on shorter autoradiographic exposures of the same samples revealed that ethanol exposure elevated type II collagen and aggrecan transcript levels in the mandibular cultures by 1.9- and 2.4-fold, respectively). Subsequent hybridization of this same blot with ^{32}p-labeled oligo $(dT)_{18}$ (not shown) confirmed that samples from ethanol-treated cultures and their corresponding controls contained near-equivalent amounts of total poly(A)$^+$ RNA.*

subridge mesenchyme of the stage 24/25 chick embryo limb bud (Kulyk and Hoffman, 1996), which contains no myogenic progenitor cells (Newman *et al.*, 1981; Gay and Kosher, 1984). It should be noted that whereas chondrogenic mesenchyme of the limb buds derives from somatic mesoderm, the entire facial skeleton differentiates from neural crest ectoderm. Thus ethanol's stimulatory influence on chondrogenesis may be a property common to all embryonic cartilage progenitor cells, regardless of their specific ontogenetic lineage. In previous studies we found that the limb and facial mesenchymes also respond similarly to staurosporine, a protein kinase inhibitor that enhances chondrogenic differentiation *in vitro* (Kulyk, 1991; Kulyk and Reichert, 1992). It is likely that there are many commonalties in the molecular pathways regulating morphogenesis and differentiation in the limb and facial skeletons, despite the differing origins of their skeletogenic progenitor cells (Helms *et al.*, 1997).

There is extensive literature documenting the diverse anatomical malformations induced by ethanol exposure during vertebrate embryogenesis (Streissguth *et al.*, 1980; Webster, 1989; Schenker *et al.*, 1990; Becker *et al.*, 1996). These include specific aberrations of skeletal, cardiac, urogenital and central nervous system structures, as well as general growth retardation. Ethanol's teratogenic effects have usually been attributed to generalized cytotoxicity or to systemic disturbances of nutrient uptake, placental transport, cell proliferation, and essential metabolism (Streissguth *et al.*, 1980; Sulik *et al.*, 1981; Webster, 1989; Schenker *et al.*, 1990; Kotch and Sulik, 1992; Kotch *et al.*, 1992; Zajac and Abel, 1992; Henderson *et al.*, 1995; Shibley and Pennington, 1997). However experimental investigations of ethanol effects on embryogenesis have traditionally employed *in vivo* systems or whole embryo cultures, where it is difficult to resolve systemic influences from selective effects on individual target cell populations. The high incidence of facial dysmorphologies associated with gestational ethanol exposure in both humans and laboratory animals (Webster and Ritchie, 1991; Weston *et al.*, 1994) suggests that the embryonic facial primordia possess a relatively acute sensitivity to alcohol-mediated perturbations of growth and differentiation. Indeed, recent investigations suggest that ethanol exposure preferentially depresses the viability and/or motility of cranial neural crest cells at the time they migrate from the converging neural folds into the developing facial primordia (Cartwright and Smith, 1995a,b; Rovasio and Battiato, 1995; Chen and Sulik, 1996). Our current findings suggest the possibility that ethanol might, in addition, influence the extent of chondrogenic differentiation of neural crest cells within the facial primordia. However, further investigations will be required to ascertain whether ethanol's striking effects on facial cartilage differentiation *in vitro* bear a physiological relationship to the mechanisms underlying alcohol's teratogenic actions on facial morphogenesis *in vivo*.

In our study, maximal stimulation of chondrogenesis in cultures of embryonic facial mesenchyme occurred at concentrations of 1-1.5% ethanol (approximately 175-260 mM), although statistically significant increases in sulfated GAG deposition were induced by as little as 0.5% ethanol. Although approaching the level of lethality *in vivo*, blood alcohol concentrations in this range may by attained and tolerated by human alcoholics (Lindblad and Olsson, 1976; Davis and Lipson, 1986). Indeed, relatively high doses of ethanol (approximately 85-175 mM) are necessary to reproducibly induce congenital facial malformations and other FAS-type birth defects in

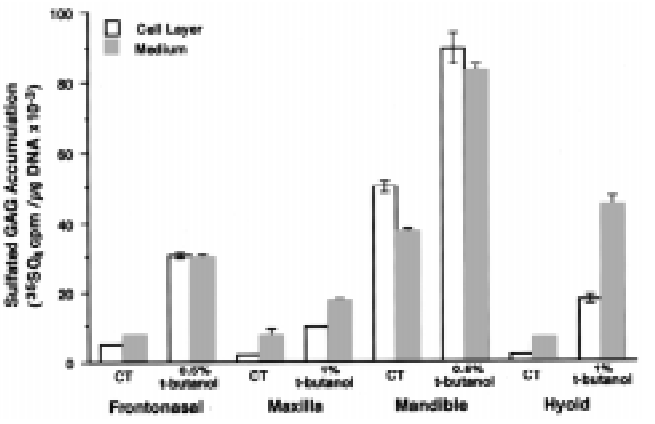

Fig. 5. Levels of $^{35}SO_4$-labeled GAG accumulation in the cell layer and culture medium of 3-day micromass cultures of embryonic chick facial mesenchyme cells incubated in the absence (CT) or presence of either 0.5% *t*-butanol (frontonasal, mandible) or 1.0% *t*-butanol (maxilla, hyoid). *Error bars represent the mean ± standard error of determinations from four replicate cultures. (Error bars that are not visible lie within the dimensions of the data point illustrated). In all facial cultures, t-butanol treatment induced significant elevations in both pericellular and medium-associated GAG accumulation (P<0.05; ANOVA, Bonferroni's multiple comparisons test).*

laboratory rodents (Webster *et al.*, 1980, 1983; Sulik *et al.*, 1981; Webster, 1989; Webster and Ritchie, 1991). Thus the range of ethanol concentrations that are effective in stimulating *in vitro* chondrogenesis in the present study does appear to overlap the dose range required to experimentally induce facial malformations *in vivo*.

We speculate that if ethanol can elevate chondrogenic differentiation *in vivo* as it does *in vitro*, then malformations might arise through the induction of precocious, excessive, or ectopic cartilage formation within the facial primordia. The vertebrate facial skeleton develops in a complex fashion, through the formation of permanent and/or transient cartilaginous structures from the facial mesenchyme at some sites and through direct intramembranous ossification at others. Even slight perturbations in the relative extents of chondrogenic versus osteogenic differentiation in the developing facial primordia might conceivably lead to the types of subtle facial dysmorphologies that are most commonly associated with prenatal alcohol exposure (hypertelorism, deficient philtrum, low nasal bridge, clefting of the lip and/or palate, etc.). Interestingly, whereas ethanol exposure is more frequently associated with inhibitory effects on cell proliferation, viability, and metabolism (Shibley and Pennington, 1997), there have been other recent reports of alcohol enhancing differentiation in specific embryonic progenitor cell populations. Ethanol exposure *in ovo* appears to alter cell fates in the embryonic chick brain, enhancing the differentiation of GABAergic neurons (Kentroti and Vernadakis, 1992) and increasing numbers of differentiated astrocytes (Srivastava *et al.*, 1995). In addition, alcohol accelerates the development of early cleavage-stage mouse embryos *in vitro* (Leach *et al.*, 1993).

The molecular mechanisms that mediate ethanol's striking stimulatory influence on *in vitro* chondrocyte differentiation remain to be defined. Experiments on a variety of cell types have demonstrated that alcohols can alter the fluidity of biological membranes (Taraschi and Rubin, 1985) and affect critical membrane-associ-

ated signal transduction events (Littleton, 1989). Ethanol exposure can directly or indirectly modulate interactions between signaling ligands and their receptors (Henderson *et al*., 1989; Resnicoff *et al*., 1993; Tomono and Kiss, 1995), phosphatidylinositol turnover (Rooney *et al*., 1989; Kiss and Garamszegi, 1993), G protein activation and intracellular protein kinase activities (Messing *et al*., 1991; DePetrillo and Liou, 1993; Slater *et al*., 1993; Reddy and Shukla, 1996), adenylate cylase activity and cAMP levels (Weston and Greene, 1991; Nagy and DeSilva, 1992; Rabin *et al*., 1992), intracellular calcium levels (Littleton *et al*., 1991; Rout *et al*., 1997), arachidonate metabolism and prostaglandin production (Szabo *et al*., 1992; Balsinde, 1993; Navamani *et al*., 1997). In addition, alcohols may disrupt retinoid biosynthesis (Deltour *et al*., 1996) and expression of the *Msx*-2 homeobox gene (Rifas *et al*., 1997), which play regulatory roles in tissue patterning and apoptosis during development. Virtually all these molecular pathways have been implicated in the regulation of embryonic cartilage formation at some level (discussed in Kulyk and Hoffman, 1996). Thus there are a multiplicity of routes through which ethanol might conceivably influence chondrogenic differentiation in the facial mesenchyme. Our future work will focus on elucidating the mechanisms whereby ethanol stimulates *in vitro* chondrogenesis and will explore the relationship of its *in vitro* actions to alcohol's *in vivo* teratogenicity.

Materials and Methods

Cell cultures

Maxillary, mandibular, frontonasal, and hyoid facial processes were dissected from stage 24 chick embryos (Hamburger and Hamilton, 1951) as previously described (Wedden *et al*., 1986; Kulyk and Reichert, 1992). As previously detailed (Kulyk and Reichert, 1992), the isolated facial primordia were stripped of surface ectoderm following dispase treatment, incubated in 0.25% trypsin, and dissociated into a suspension of $2x10^7$ cells/ml in DMEM:F12/10% FBS medium (a 1:1 mixture of DMEM and Ham's F12 medium containing 10% fetal bovine serum, 14 mM Hepes, pH 7.2, 2 mM glutamine and antibiotics). Superconfluent micromass cultures of facial mesenchyme were established by spotting 10 µl drops of cell suspension ($2x10^5$ cells/10 µl) onto plastic tissue culture plates (35 mm NUNC dishes; 4- or 24-well NUNC plates). After incubation for 1.5-2 h at 37°C, 5% CO_2 to permit cell attachment, the plates were flooded with DMEM:F12/10% FBS medium. For alcohol treatments, the medium was supplemented with reagent grade ethanol or tertiary butanol (*t*-butanol; 2-methyl 2-propanol) to a final concentration of 0.5-2.0% (v/v). Control cultures were supplemented with an equivalent volume of sterile distilled water. The plates were sealed with parafilm to minimize evaporation and incubated for up to 3 days at 37°C, 5% CO_2 without medium change.

Histochemistry

To demonstrate the deposition of cartilage matrix proteoglycans, representative cultures were collected at 3 days of incubation and stained with 0.5% Alcian blue 8GX, pH 1 (Hassel and Horigan, 1982).

Analysis of sulfated glycosaminoglycan (GAG) accumulation

To quantify relative levels of cartilage matrix GAG accumulation, the medium of some cultures was supplemented with 5 µCi/ml $[^{35}S]H_2SO_4$ at the start of the culture period. Following 3 days of continuous labeling, sulfated GAG was isolated from the both the cell layer and culture medium by precipitation with cetylpyridinium chloride as previously described (Kosher, 1976; Kulyk *et al*., 1989). The GAG-incorporated label was quantified by liquid scintillation counting. Total cellular DNA was determined by fluorometry (Brunk *et al*., 1979). The data were analyzed statistically using InStat 2.01 software for Macintosh (GraphPad Inc.).

RNA analysis

Total RNA was isolated from pooled cells of 5-10 micromass spot cultures (approximately $2\text{-}4x10^6$ cells) using TriZOL Reagent (Gibco/BRL) according to the manufacturer's directions. The relative poly(A+) RNA abundance of each RNA sample was determined by dot-blot hybridization with ^{32}P-labeled oligo$(dT)_{18}$ (Harley, 1987). Steady-state levels of type II collagen and aggrecan (cartilage proteoglycan) mRNA transcripts were determined by dot-blot hybridization with ^{32}p-labeled cDNA probes under stringent conditions exactly as previously described (Kosher *et al*., 1986a,b; Kulyk, 1991). The chicken α1(II) collagen probe was pCAR2, a 680bp cDNA in pBR322 (Vuorio *et al*., 1982), obtained from W.B. Upholt, University of Connecticut Health Center. The chicken aggrecan probe was ST-1, 1.2 kb cDNA in pUC9 (Sai *et al*., 1986), provided by M.L. Tanzer, University of Connecticut Health Center. The specificities of these probes in dot-blot RNA analysis were verified in earlier studies (Kosher *et al*., 1986a,b). Following hybridization, the dot-blots were exposed to Reflection (Dupont/NEN) autoradiography film at -80°C. Hybridization signals were quantified by measuring absorbance at 540 nm using a Tecan SLT Spectra II spectrophotometric plate reader.

Acknowledgments

We are grateful to Drs. W. Upholt and M.L. Tanzer, University of Connecticut Health Center, for generously providing the pCAR2 and ST-1 cDNA probes used in our study. This research was funded by an operating grant to W.M.K from the Natural Sciences and Engineering Research Council of Canada. L.M.H. was supported by a scholarship and teaching fellowship from the University of Saskatchewan College of Graduate Studies.

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Received: September 1998
Accepted for publication: October 1998