

EFFECTS OF ETHANOL ON GLIAL CELL PROLIFERATION: RELEVANCE TO THE FETAL ALCOHOL SYNDROME

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1. ABSTRACT

Exposure to ethanol during pregnancy is detrimental to brain development. Individuals affected by the Fetal Alcohol Syndrome present a number of central nervous system dysfunctions including microencephaly and mental retardation. Studies on the mechanisms of ethanol's developmental neurotoxicity have focused on its interaction with neurons; however, emerging evidence is suggesting that ethanol can significantly affect glial cells as well. A number of *in vitro* studies have shown that ethanol can inhibit the proliferation of various glial cells (mostly primary astrocytes or astrocytoma cells) at relatively high concentrations (100-200 mM). On the other hand, proliferation induced by some, but not all mitogens, is inhibited by low concentrations (10-50 mM) of ethanol. These inhibitory effects of ethanol may contribute to its developmental neurotoxicity observed following *in vivo* exposure. Animal models have indeed shown that ethanol causes microencephaly when given during the brain growth spurt, a period of brain development characterized by astroglial proliferation and maturation.

2. INTRODUCTION

Offspring of alcoholics often present a syndrome (Fetal Alcohol Syndrome or FAS) whose principal features include central nervous system (CNS) dysfunctions (mental retardation, microencephaly, brain malformations), growth deficiency, and particular facial features (1). The CNS deficits of FAS appear to be long lasting, since recent studies have shown that they persist in young adults born with FAS, even if other symptoms (growth retardation, facial characteristics) have subsided (2-4). FAS is now considered a leading cause of

mental retardation in the general population (5), and a recent report indicates that its incidence has increased over six-fold in the last 15 years, from 1 per 10,000 births in 1979 to 6.7 per 10,000 births in 1993 (6).

A large number of studies have been conducted in laboratory animals to gain an understanding of the characteristics and mechanisms of alcohol teratogenicity, and their results have been summarized in several books and reviews (7-10). *In vivo* studies in rodents have utilized both prenatal and postnatal exposures, to mimic human exposure during the first and early second trimester of pregnancy, and the late second and third trimester and early postnatal life, respectively. As the development of the nervous system differs across species (11), and since, within one species, different brain regions and cell types develop at different times, it has become apparent that the timing of exposure to ethanol is extremely important for specific adverse effects to be manifest. When ethanol is administered during the brain growth spurt (the first two postnatal weeks in the rat, characterized by proliferation of glial cells and maturation of neurons which develop dendrites, axons and synaptic contacts), the most striking effect observed is microencephaly (12), which is also seen in 80% of FAS children, and appears to be irreversible in both animals and humans (3,4,13,14).

The majority of studies aimed at exploring the neurologic sequelae resulting from alcohol abuse during pregnancy have focused on neuronal susceptibility. Several investigations have shown that exposure to ethanol can cause, for example, selective losses of hippocampal pyramidal cells or cerebellar Purkinje cells (15-17). A number of studies on the morphological and behavioral consequences of developmental exposure to ethanol have also been published, and will not be reviewed here. The extent to which glial cells, the intimate neighbors of neurons, are vulnerable to ethanol, has been much less investigated. Yet, in recent years, evidence has been accumulating that ethanol can significantly affect glial

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cells (18-20). A number of effects of ethanol on glial cells biochemistry and metabolism, morphology and differentiations have been found, and have been recently reviewed (20).

The present review will focus on the effects of ethanol on the proliferation of glial cells and will discuss the hypothesis that interference with this process may play a relevant role in the developmental neurotoxicity of alcohol.

3. EFFECT OF ETHANOL ON GLIAL CELL PROLIFERATION

More than a decade ago, Davies and Vernadakis (21) examined the effect of exposure of glial cells, prepared from 15-day-old chick embryos, to ethanol (22, 109, 217, and 434 mM) for four days (day 6 to 10 in culture). The two higher concentrations of ethanol caused a 20-40% reduction in DNA content, while at the lower concentrations cell growth did not differ from control. These observations were confirmed in a subsequent study, where exposure of rat mixed glial primary cultures to ethanol (17, 43, and 86 mM) for four days had no effect on DNA synthesis (22). On the other hand, Kennedy and Mukerji (23) reported that astrocytes from newborn mice cultured from postnatal day 6 to 17 in the presence of ethanol (11, 22, and 45 mM) displayed a decrease content of DNA compared to untreated controls; the effect was quite small and was seen only at the highest alcohol concentration (45 mM). Yet, in another study, no effects of ethanol (10-60 mM) were observed following a four day exposure of fetal rat astrocytes (24).

In two additional studies (25,26) ethanol (44, 109, and 217 mM) was found to inhibit the growth of rat cerebral glial cultures following an exposure for seven days or longer. Inhibition of [³H]-thymidine incorporation by a 28 day exposure of rat cortical astrocytes to 100 mM ethanol was also reported (27). Similarly, 100 or 200 mM ethanol were found to inhibit [3H]-thymidine incorporation of rat astrocytes following a 18-24 hr exposure (28,29), though no effects were seen in another study (30). The proliferation of astroglia from adult human cerebrum, measured by 5-bromo-2'-deoxyuridine-5'-monophosphate incorporation into DNA, was also inhibited by ethanol (22, 45, and 109 mM) (31).

Two studies also examined the effect of ethanol on proliferation of C6 glioma cells (measured by cell counting; 32,33) and found contrasting results. In one case, inhibition of proliferation was found, at ethanol concentrations of 30-120 mM, while in the other no effect of ethanol at 80 mM was found (33).

Altogether, these findings consistently indicate that ethanol can inhibit the proliferation of glial cells *in vitro*. Though in some studies ethanol exerted an effect at concentrations below 100 mM, most studies found that

high concentrations of ethanol (>100 mM) were necessary for significant inhibitory effect on proliferation to be manifest. The reason for these quantitative differences are not clear, but may be related to the different systems utilized; for example, Kane *et al.* (31) observed a small (10%) inhibition of proliferation by 22 mM ethanol in astrocytes derived from human adult temporal lobe, while all other studies made use of fetal or neonatal astrocytes from rodent brain. In all these studies, cells were cultured in medium containing serum and concomitantly exposed to ethanol. Under these conditions, cells are expected to be divided among the G0/G1, S and G2/M phases of the cycle, and the inhibitory effect of ethanol may be related to its ability to produce a block in the G0/G1 phase (34). Indeed, when human astrocytoma cells were serum deprived, so that >98% were in G0/G1, no effect of ethanol were measured by flow cytometry (35). In this case, the observed inhibition of [³H]-thymidine incorporation by 100-200 mM ethanol could be attributed to nonspecific effects on thymidine uptake, or to other interferences with cellular metabolism (e.g. DNA repair) (35).

A major metabolite of ethanol is acetaldehyde, which has been shown to inhibit cell growth and induce apoptosis in other cell types (e.g., the Chinese hamster ovary cell line A-10; 36). However, evidence suggests that ethanol itself, and not acetaldehyde, may be responsible for the observed inhibitory effect on cell proliferation. Astrocytes lack alcohol dehydrogenase, which catalyzes the conversion of ethanol to this metabolite (37), and the lack of this enzymatic pathway was also suggested in human astrocytoma cells (35). Furthermore, co-incubation of ethanol with 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, failed to affect the inhibitory action of ethanol (32, 35). Thus, ethanol, at high concentrations, appears to be able to inhibit proliferation of astrocytes or glioma cells, without the need for metabolic activation.

4. EFFECT OF ETHANOL ON MITOGEN-STIMULATED GLIAL CELL PROLIFERATION

Only a limited number of studies have examined the effect of ethanol on the proliferation of glial cells stimulated by mitogens. Overall, ethanol appears to be a potent, but selective, inhibitor of stimulated proliferation, as it is capable of completely blocking the action of certain mitogens at concentrations of 50-100 mM, while it is ineffective toward other mitogens.

In a study by Luo and Miller (33) the proliferation of C6 rat glioma cells induced by basic fibroblast growth factor (bFGF) was measured by cell counting and by [³H]-thymidine and bromodeoxyuridine incorporation. Incubation with 80 mM ethanol caused a total block of cell proliferation and the IC₅₀ was about 20 mM (33). These results differ from those of de Vito *et al.* (29) who reported a lack of effect of ethanol (50 mM) on [³H]-thymidine incorporation in rat cortical astrocytes

Table 1. Effect of Ethanol on Mitogen-Stimulated Glial Cell Proliferation

MITOGEN	EFFECTIVE ETOH CONCENTRATIONS (mM)	REFERENCE
IGF-I	10-100	38, 39
EGF	10-100	38, 39
bFGF	20-80	33
Muscarinic agonists	5-100	35
Prolactin	10-60	29
Histamine	5-100	35
Interleukin-1	5-100	35
Phorbol esters	5-100	35
Insulin	no effect	35
PDGF-BB	no effect	29,35,39
FGF	no effect	29

induced by FGF. These authors, however, reported that ethanol inhibited the proliferation of astrocytes induced by prolactin, with an IC_{50} of about 25 mM (29). Interestingly, inhibition of prolactin-induced [3H]-thymidine incorporation was seen not only when ethanol and the mitogen were co-incubated, but also when cells had been exposed to ethanol for at least 6-18 hours prior to the addition of prolactin. A decrease in prolactin-induced astrocyte proliferation was also found in astrocytes prepared from rats that had been prenatally exposed to ethanol (5% ethanol in drinking water for the last five days of gestation). Thus, it would appear that the presence of ethanol together with this mitogen is not necessary for its inhibitory effect to be manifest, and that prolonged exposure to ethanol may cause changes in the cells that prevent the action of this mitogen.

In another study, ethanol was found to be a potent inhibitor of proliferation of rat cortical astrocytes and human astrocytoma cells induced by muscarinic agonists (carbachol, methacholine, acetylcholine, bethanecol), with an IC_{50} of about 10 mM (35). Inhibition of carbachol-stimulated proliferation of glial cells was of the noncompetitive type, and was observed by measuring [3H]-thymidine incorporation into DNA, as well as by flow cytometry. Using this latter technique (which utilizes 5-bromodeoxyuridine and Hoechst 3325), carbachol (0.1 mM) was found to increase the percent of cells in S/G2 from 0.83 to 23.3. Ethanol (25 mM) had no effect when present alone, but significantly decreased the effect of carbachol (to 12.3% of cell in S/G2 phase) (35).

Resnicoff *et al.*, have examined the effect of ethanol on proliferation of rat C6 glioblastoma cells induced by insulin-like-growth-factor I (IGF-I); alcohol completely inhibited proliferation (determined by cell counting with a hemocytometer) with an IC_{50} of 10-25 mM (38,39).

Though these four mitogens have been investigated more in detail with regard to their sensitivity to ethanol, a few others have also been examined (Table 1). Of notice is that ethanol was ineffective in inhibiting proliferation of glial cells induced by insulin and platelet-derived-growth factor (PDGF), while it was a potent inhibitor of the mitogenic action of the phorbol ester 12-O-

tetradecanoylphorbol 13-acetate, TPA), with an IC_{50} of 15.6 mM (35).

Little is known, so far, on the mechanism(s) involved in ethanol's inhibition of mitogen-stimulated proliferation. In case of IGF-I, it has been shown that the receptor of this growth factor undergoes tyrosine-autophosphorylation of the beta-subunit upon stimulation with the agonist, and this process was completely inhibited by 100 mM ethanol (38). This mechanism may also account for ethanol's inhibition of EGF (epidermal growth factor)-induced proliferation; the latter has indeed been shown to be due to induction of IGF-I receptors by EGF, and a subsequent mitogenic action mediated by an IGF-I/IGF-I receptor autocrine pathway (39).

Ethanol has been shown to inhibit muscarinic receptor-coupled second messenger systems in glial cells, including formation of inositol 1,4,5-trisphosphate, mobilization of intracellular calcium, and activation of phospholipase D (40; Catlin and Costa, unpublished). However, the relative roles of these intracellular pathways in the inhibition of carbachol-stimulated proliferation by ethanol, is still not clear. The finding that proliferation induced by TPA is inhibited by ethanol with high potency has led to the hypothesis that protein kinase C (PKC) may play a role in the inhibitory effect of alcohol (35). However, the reported effects of ethanol on PKC isozymes seem to differ (i.e., stimulation vs. inhibition) depending on the alcohol concentration, the cell type and the mode of exposure (i.e., acute vs. chronic). Thus, substantial additional research is needed to test this hypothesis. The ability of ethanol to potently inhibit mitogen-induced proliferation of glial cells is in agreement with its reported ability to block cells in the G0/G1 phase (34). Still, to be understood, however, is why some mitogens are not sensitive (or less sensitive) to the inhibitory action of ethanol.

5. INHIBITION OF GLIAL CELL PROLIFERATION AND ETHANOL'S DEVELOPMENTAL NEUROTOXICITY

As mentioned in the introduction, one of the main findings in individuals diagnosed with the Fetal Alcohol Syndrome, is the presence of microencephaly (1-4, 14). Animal models of FAS have shown that microencephaly is observed when ethanol is given during the first two postnatal weeks in the rat, a period corresponding to the third trimester of pregnancy in humans (12, 13, 41). This period of brain development corresponds to the so called "brain growth spurt," which is characterized by proliferation of glial cells and synaptogenesis (11). Thus, it is plausible to hypothesize that the effect of ethanol on glial cell proliferation may play a relevant role in its developmental neurotoxicity, most notably microencephaly.

There is evidence that developmental exposure to ethanol *in vivo* may affect the development and

migration of glial cells (18, 42), and cause a reduction in cell number (43, 44). Microencephaly has been produced in rats following *in vivo* exposure from postnatal day 4 to 10 to ethanol doses which produced blood alcohol concentrations (BAC) of about 50 mM (45). Other investigators also found microencephaly following similar ethanol exposures and BACs of about 40 mM (41, 46). At these concentrations, ethanol has limited or no effect on the *in vitro* proliferation of glial cells cultured in the presence of serum. However, as discussed above, at concentrations of about 50 mM, ethanol has been shown to profoundly inhibit the proliferation induced by several mitogens. In case of acetylcholine- and IGF-I-induced proliferation there is initial evidence that the inhibitory effect of ethanol, observed so far only *in vitro*, may be relevant to *in vivo* situations.

There is growing evidence that acetylcholine may influence various aspects of brain development (47). Of particular interest is that acetylcholine levels are particularly high in the neonatal rat brain (80-90% of adult values), and that muscarinic receptor-activated signal transduction systems (notably phospholipases C and D) are greatly enhanced in the neonatal rat brain compared to adults (40, 48), despite a lower receptor density. A good correlation has been found between the ability of ethanol to induce microencephaly, and its ability to inhibit muscarinic receptor-stimulated phosphoinositide metabolism (45, 49). Additionally, as discussed earlier, muscarinic agonists can cause proliferation of rat cortical astrocytes and their action is significantly inhibited (about 80%) by 50 mM ethanol. Thus, during the brain growth spurt, acetylcholine, by activating muscarinic receptors coupled to phospholipase C (e.g. the m_3 subtype, which is expressed in astrocytes; 50) may contribute to the proliferation of astrocytes which occurs during this period (51). This process may be a target for ethanol, and may underlie, at least in part, the microencephaly observed following developmental ethanol exposure.

A similar set of considerations may also apply to IGF-I, though less evidence is so far available. This growth factor and its receptor appears to play a significant role in development, including the development of the nervous system (52). In addition to the aforementioned *in vitro* inhibition by ethanol of IGF-I-induced proliferation of C6 glioma cells, there is also evidence that *in vivo* administration of alcohol to pregnant rats (yielding BAC of about 13 mM), causes a decrease in circulating levels of IGF-I (53), by still unknown mechanisms. Thus, in this case, ethanol would alter the levels of IGF-I, in addition to directly inhibiting its mitogenic action in astrocytes.

In summary, several *in vitro* studies have shown that alcohol can inhibit the proliferation of glial cells (either astrocytes or transformed cell lines). The effect of ethanol is more pronounced toward proliferation induced by mitogens, and occurs at concentrations (20-50 mM) which correspond to moderate to high drinking. The

action of ethanol also appears to have some degree of specificity, as only the effect of certain mitogens is affected. The ability of ethanol to interfere with the mitogenic action of certain neurotransmitters and growth factors (e.g., acetylcholine or IGF-I) in glial cells, may explain some aspects of the developmental neurotoxicity of alcohol, most notably microencephaly. When exposure to ethanol occurs at different stages of brain development (e.g. during the first or second trimester equivalent in human), inhibition of neuronal proliferation may also occur. Indeed, ethanol has been shown to inhibit proliferation of cells other than glia, including neurons, hepatocytes, lymphocytes and osteoblasts (54-57).

These combined effects on CNS cell proliferation, together with possible direct or indirect toxic effect of ethanol on developing neurons, may provide a comprehensive picture of the events responsible for CNS dysfunction in FAS patients.

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