**PHARMACOLOGY AND CELL METABOLISM**

**ALCOHOL EXPOSURE ALTERS CELL CYCLE AND APOPTOTIC EVENTS DURING EARLY NEURULATION**

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Abstract — Background: Fetal alcohol exposure causes growth deficits, microencephaly, and neurological abnormalities. Although the effects of alcohol on developmental delay and growth-related deficits have been hypothesized, little is understood about how alcohol alters, in particular, the cyclin pathway within the cell cycle, which is critical to proliferation and apoptotic control. In this study, we examined cell cycle proteins pertinent to the G1–S phase transition and apoptosis, to determine if cell cycle misregulation can be attributed to apoptotic induction and growth defects. Methods: We examined cell cycle regulation during G1 and S-phase, and DNA fragmentation damage, using E14 dorsal root ganglia neural stem cells (DRG-NC), and cultured mouse embryos exposed to 200 and 400 mg/dl ethanol. Results: Alcohol-exposed DRG-NC demonstrated a dose-dependent increase in cells expressing increased cyclin D1 protein, and increased DNA fragmentation. Western blot analysis, using embryos, demonstrated an overexpression of cyclin D1, D2, and E2F1, key G1 to S-phase cell cycle regulatory components, and increases in p53, linking the cell cycle and apoptotic pathways. Bromodeoxyuridine incorporation indicated reduced DNA synthesis and growth in several embryonic regions. Propidium iodide staining demonstrated decreases in DNA content and increases in DNA fragmentation in several embryonic tissues. Conclusions: This study indicated that retarded growth of DRG-NC and embryos, induced by alcohol, is associated with altered expression of cell cycle and apoptotic proteins and concurrent inhibition of proliferation and increased DNA fragmentation. We suggest that alcohol induces an increase in cyclin D1 expression, premature S-phase entry, and disjointed DNA synthesis with increased apoptosis.

INTRODUCTION

Children born to women who drink heavily during pregnancy are at risk for developmental disorders, the most prominent being fetal alcohol syndrome (FAS), in which the affected child suffers from growth retardation, central nervous system damage, and a characteristic pattern of abnormal facial features (Jones and Smith, 1973; Clareen et al., 1978; Sampson et al., 1997; Kalter, 2003). It is now recognized that a greater range of phenotypic structural and growth deficits can result from prenatal alcohol exposure, now referred to as fetal alcohol spectrum disorders (FASD) (Streissguth and O’Malley, 2000; Autti-Ramo et al., 2006) in which growth retardation is a key element. Multiple pathways can lead to ethanol’s effect on growth retardation, including reduced proliferation and accelerated cell death (for a recent review see Goodlett et al., 2005). Changes in cell proliferation (Luo and Miller, 1998) involving altered cell cycle regulation (Miller and Kuhn, 1995), disrupted DNA and protein synthesis (Shibley and Pennington, 1997), and apoptosis (Kotch et al., 1992; Ewald and Shao, 1993; Holownia et al., 1997; Zhang et al., 1998; Ikonomidou et al., 2000; Climent et al., 2002; Light et al., 2002) possibly contribute to the growth defects and the microencephaly characteristic of FASD.

During normal cell cycle progression, growth phase 1 (G1 phase) cells increase the expression of proteins necessary to direct the later DNA synthesis phase (S-phase). During mid-G1, a transient increase of cyclin D1 and D2 protein expression initiates a cascade of events that trigger the cell to pass the restriction point and proceed to S-phase, in part, by phosphorylation of the retinoblastoma protein (pRB) and activation of E2F1 transcription of genes required for proper DNA synthesis and chromatin condensation (for review see Ekholm and Reed, 2000; Tan et al., 2006). Individual overexpression or misregulated expression of cyclin D1 or E2F1 has been shown to induce uncontrolled cell cycle progression leading to altered DNA synthesis proteins and induced apoptosis (Kowalik et al., 1995; Sofer-Levi and Resnitzky, 1996; Merino and Cordero-Campana, 1998; Hiyama and Reeves, 1999). A fundamental cell cycle regulator, p53, has been reported to be a link between this misregulated cell cycle control and apoptosis, by way of phosphorylation of p53 in part at serine 20 (Cordon-Cardo, 1995; Enoch and Norbury, 1995; Johnson and Schneider-Broussard, 1998; Caspari, 2000).

Currently, it is unclear how alcohol affects the cell cycle. An important step towards understanding cell cycle and apoptosis-related, alcohol-induced, growth deficits in FASD, is to identify what changes in protein expression occur during distinct cell cycle phase regulation. Our previous microarray analysis, using an embryo culture model, identified expression alterations in gene sets that included cell cycle regulators in G1 and S-phase, DNA excision and repair, and apoptosis (Zhou et al., 2001). This gene set included cyclins (D1, D2), E2F1, and p53, all of which could have profound consequences on proper proliferation and organogenesis.

In the current study, we adopted a mouse embryonic dorsal root ganglia neural stem cell (DRG-NC), in which proliferation can be closely monitored and synchronization can be tightly controlled by plating and medium conditions. We also used an established whole embryonic culture system (Ogawa et al., 2005) to examine cell cycle and apoptotic alterations in specific organs. Our aim in this study was to examine the changes in G1 and S-phase protein expression and begin to delineate how alterations in cell cycle regulation and apoptosis may contribute
to growth defects induced by alcohol. This information will be helpful to begin delineation of the mechanism(s) involved in growth related defects in FASD.

This study demonstrated that a collective misregulation of protein expression, including cyclin D1, D2, E2F1, and p53, pivotal to regulation of the G1 phase of the cell cycle, premature induction of DNA synthesis (S-phase), and apoptosis occurred. Our study further demonstrated that many developing tissues that demonstrated alterations in cyclin D1 expression also demonstrated increased DNA fragmentation and apoptosis.

MATERIALS AND METHODS

E14 dorsal root ganglia cell cultures

Cells were cultured in DMEM/F12 with 10 units/ml Fungizone, 20 units/ml penicillin and 20 units/ml streptomycin (Sigma, St. Louis, MO), 10 ng/ml EGF and bFGF. Cells were triturated to single cell suspension and plated at 2.0 × 10^5 cells/ml in 24 well plates (Corning) in 2 ml of fresh media. After 24 h of culture, cells were exposed to 400 and 200 mg/dl alcohol and cultured for 48 h. Alcohol concentration analysis was performed at 0, 24, and 48 h using a gas chromatograph calibrated to 0–500 mg/dl. Each experiment was run in triplicate. Statistical analyses were carried out using the Statview software. Fisher’s Student’s t-tests were performed with P ≤ 0.05.

DRG-NC proliferation

To determine cell proliferation, a 250 ml sample of cultured cells were extracted from each treatment group at 0, 6, 12, 24, and 48 h. Cells were centrifuged at 4000 rpm and pellets were collected and suspended in 20 ml PBS/1% Trypan blue, and incubated for 15 min at RT. Two separate 10-ml samples were used for cell counts using a hemocytometer. The sample times for each treatment group were assayed in triplicate. Cell counts are presented as mean ± SEM for each treatment group and time, and as a percentage of the starting cell counts, set at 100%. Statistical analyses included Fisher’s Student’s t-test (P < 0.05); the Statview software was used.

Immunohistochemistry and propidium iodide staining for DRG-NC. Cultured cells were washed in PBS then fixed overnight in 4% PFA. Cells were collected by centrifugation at 4000 rpm to collect the cell pellet following each step. Cells were incubated in MOM kit per manufacturer’s specifications (Immunodetection Kit, Vector Laboratories, Burlingame, CA) to block nonspecific primary antibody binding, washed in PBS, then incubated in MOM working solution containing 1/100 dilution of monoclonal cyclin D1 antibody (Cell Signaling Tech.) for 24 h at 4°C. Cells were rinsed two times with PBS and incubated with a goat antimouse Alexafluor 350 antibody (Molecular Probes) at a dilution of 1/100 for 3 h at room temperature, washed with PBS and equilibrated in 2 × SSC for PI counterstaining. Cells were incubated for 20 min in 2× SSC with 20 units of DNase-free RNase in 50 ml (Promega), washed in 2× SSC and incubated for 30 min in a 1 mg/ml solution of propidium iodide, then washed again in 2× SSC. Cells were re-suspended in PBS, placed on slides, cover-slipped, and used for cell counts and microscopy.

For apoptotic analysis, a total of three independent cultures were used for each treatment group, counted in triplicate, with >10^4 cells for each cell count. For each treatment group, samples were collected at the start of the culture, and at 6, 12, 24, and 48 h. Cells were stained for PI only and counted for (1) total cells with PI staining and (2) the number of cells with fragmented, pyknotic foci (apoptotic indicator), and calculated as the number of apoptotic cells per total cell count. Data are presented as the percentage of apoptotic cells with the initial starting culture (time 0) set at 100%. Statistical analysis used Student’s t-test for P < 0.05.

Cell counts for both PI and D1 dual labeling used a total of three independent cultures, counted in triplicate, for each treatment group, with >10^3 cells from each cell count. For cyclin D1, three populations of cells were counted: (1) All cells (determined by any PI staining), (2) All cells with low D1 expression (described as light diffuse cytoplasmic blue fluorescence) and (3) All cells with high expression (this was seen as bright blue focal fluorescence in both cytoplasmic and nuclear regions).

Dual-labeled cells were also counted for total, high, or low PI staining. Two additional cell counts were also determined. First, cells with fragmented DNA and reduced (low) PI staining (determined by pyknotic cells with little compact DNA), and second, cells with DNA fragmentation that showed an increased (high) PI staining (described as disrupted DNA foci with >4N DNA).

In all cell counts, data are presented as the percentage of low and high expressing cells to the total cell counts, compared to controls, which were set at 100%. Data from all counts are presented as mean ± SEM. In the statistical analyses, the Statview software was used and Fisher’s Student’s t-test with P < 0.05 was performed.

Embryonic culture

The use of animals was approved by the Institutional Laboratory Animals Research Committee at Indiana University School of Medicine, and are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996). Two-month-old C57BL/6 mice (~20 g) were purchased from Harlan, Inc. (Indianapolis, IN). Upon arrival, the breeder mice were individually housed and acclimated for at least 1 week before mating began. The mice were maintained on a 12 h light–dark cycle (light on: 19:00–7:00) and provided laboratory chow and water ad libitum. Two females were placed with one male for two hours. The mating time was 8:00–10:00. When a vaginal plug was detected after the mating period, it was designated as embryonic day 0 (E0). On E8 at 15:00, dams were sacrificed using CO2 gas.

The technique for whole embryo culture was based on the method described by New (New, 1978). Briefly, the gravid uterus was removed and placed in a sterile PBS (0.1 M phosphate-buffer-containing saline) at 37°C. The embryo in the visceral yolk sac along with a small piece of the ectoplacental cone (hereafter called embryo, unless otherwise stated) was carefully removed from deciduous tissues and the Reichert’s membrane and immediately immersed in PBS containing 4% fetal bovine serum (Sigma, St Louise, MO). Three embryos bearing 3–5 somites (E8.25) were placed in a culture bottle
buffer saline (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 8 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 0.9 mM CaCl₂, 5.6 mM glucose, 0.33 mM sodium pyruvate, pH 7.4) supplemented with 20 units/ml penicillin and 20 units/ml streptomycin (Sigma, St. Louis, MO). Bottles were gassed between 0 and 22 h with 5% O₂, 5% CO₂, and 90% N₂, and between 22 and 44 h with 20% O₂, 5% CO₂, and 75% N₂ in a rotating culture system (BTC Precision Incubator Unit, BTC Engineering, Cambridge, England; 36 rpm) at 37°C. After the pre-culture period (2–4 h; max 4 h), we began the alcohol exposure began by transferring the embryos into a medium containing 6 μg/ml of 95% ethanol (reaching 400 mg/dL in culture). The culture medium was replaced with fresh medium ± ethanol 22 h after the start of the exposure. All cultures were terminated 44 h from the beginning of treatment. A total of 166 embryos—150 for Western blot, 8 for PI and D1 staining and 8 for BrdU studies, were used. For each culture, control embryos were included to avoid misevaluations produced by any differences in the cultural conditions among these experiments.

The concentration of ethanol in the medium across 24 h was tested at 0, 12, and 22 h in a separate group not used for the vulnerability study to avoid confounding variables from the sample drawing. Three media samples per each time point were tested for alcohol concentration using Analox alcohol analyzer (Analog Instruments USA, Lunenburg, MA). The target peak alcohol concentration (400 mg/dL; medium concentration curve peak at 400 mg/dL and reduced to 200 mg/dL at the end of 24 h) used in the current study is comparable to that produced by the lowest in vivo dosage of acute ethanol injections reportedly producing teratogenic effects in mice. While mice metabolize ethanol at a much higher rate than humans, this level, though high, is considered within the range attained by human alcoholics (Lindblad and Olsson, 1976).

At the end of culture, viability was confirmed by observing the blood circulation of the yolk sac and the beating heart. Depending on the assay, cultured embryos were either quick-frozen on dry ice for Western blots or fixed in 4% paraformaldehyde in PBS for the evaluation of immunocytochemical bromodeoxyuridine (BrdU) and cyclin D1, and PI staining for evaluation of cell proliferation, cell cycle progression, and apoptosis. For the cohort used in the proliferation analysis, the embryos were exposed to 5 μM BrdU in the last 2 h of culture.

**Western blot analysis**

Embryonic tissue from treatment groups were collected and lysed in RIPA (radio-immunoprecipitation assay) buffer (50 mM KCl, 50 mM Hepes (pH 7.6), 1 mM EDTA, 1 mM DTT, 5% glycerol, protease inhibitor cocktail (EDTA-free, Roche Diagnostics); 5 μl RIPA buffer per embryo). Tissue was triturated for 1 min with a pipette, vigorously vortexed, and centrifuged for 2 min at 8000rpm. The supernatant was removed and frozen at −80°C. Total protein concentrations were calculated using the Bio-Rad Colorimetric Protein Assay Kit (Bio-Rad, Hercules, CA). Standard curves were constructed using bovine serum albumin (BSA) with serial dilutions of 0.2–0.9 mg/ml. The standard curve of BSA at OD₅₉₅ was used to estimate all sample concentrations.

All Western blots followed a standard protocol (Laemmlli, 1970; Zhou et al., 1999; Zhou et al., 1996; Anthony, Zong and De Benedetti, 2001). For total protein procurement, 6 embryos were pooled as one sample and 12 control and 13 alcohol-treated samples were used in the Western blot analysis. All samples were run in triplicate on two independent gels for each protein examined. Protein extracts were run on 8–12% PAGE gels using 20–40 mg total proteins then transferred to a 45-μm nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were stained with Poncet-S (Sigma) to confound equivalent total protein loads and quality transfer. Gels were stained, after transfer, with Coomassie blue to assure complete transfer. Membranes were blocked with 5% milk and 0.2% BSA in TBST overnight, washed 3× in TBST, then incubated for 6–8 h at room temperature using the appropriate primary antibodies. Primary antibodies included: mouse cyclin D1 (Cell Signaling Tech), mouse cyclin D2 (Biosource), mAb E2F-1 (Sigma), mouse p53 (Cell Signaling Tech), and rabbit anti-p53 phosphorylated at ser20 (Cell Signaling Tech). Membranes were washed 3× in TBST and incubated with the appropriate secondary antibody conjugated to HRP, rabbit antihorse HRP, or sheep antirabbit HRP (Chemicon). Amersham Bioscience ECL Plus Western detection system (Amersham Bioscience, Piscataway, NJ) was used for HRP detection. Blots were developed using CL-Xposure x-ray film (Pierce, Rockford, IL) following the ECL plus protocol. Mouse anti-GAPDH primary antibody (Chemicon International) was used as an internal control for all Westerns. After target protein detection, membranes were stripped in a solution of 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl with pH 6.7 by incubation in a 50°C water bath for 15 min in a sealed plastic bag and re-blocked with 5% dry milk/0.2% BSA/TBST. Membranes were processed as described above using anti-GAPDH primary antibody. Each sample was run in triplicate and the average density value was taken as n = 1.

**Image analysis.** The developed x-ray films were scanned using a flat-bed scanner and density of images was measured with NIH Image Software after calibration to a 20-panel OD₉₅₅ grayscale density control, supplied by Kodak (Kodak, Rochester, NY). GAPDH density measurements were used for load controls and variations in measurements were adjusted by percentage to the highest values for that membrane. Measurements for each target protein were then measured and adjusted in accordance with the GAPDH load controls. All changes in protein expression from alcohol treatment are reported as a percentage change compared to controls with an average of >12 samples/treatment group. Statistical analysis used Student’s t-test on Statview software (SAS Institute, Inc., Cary, NC).

**Cell Cycle Genes Under Alcohol Exposure**

For detection of proliferation, the embryos were incubated in bromodeoxyuridine (BrdU, maker of DNA duplication, 5 μM) two h before termination of the culture. All embryos (n = 4 of each treatment) in this cohort were transferred through three washes of PB-1 buffer before fixation in 4% paraformaldehyde overnight. Embryos for the two treatment groups were paired and cast in gelatin together, carefully aligned by their level and orientation, then processed in parallel for immunocytochemistry from this point onwards. This practice avoids any bias...
through the staining procedure, and is convenient for comparing levels of embryo sections side by side. The paired embryos in the gel were cut into 40 μm coronal sections with a Leica 1000S vibratome. The sections were then washed three times in PBS and incubated in 3% H₂O₂ (v/v) in PBS for 30 min, and PBS containing 4% normal sheep serum (NSS) and 0.3% Triton X-100 (TX) for 30 min at room temperature to block nonspecific binding. Sections were then incubated with mouse anti-BrdU (for detection of cell division, 1:500) (Sigma-Aldrich) antibody overnight at room temperature. The secondary antibody used was biotinylated with sheep–antimouse IgG (1:250) for 1 h at room temperature. All sections were then incubated in avidin–biotin peroxidase complex (ABC, Vector Labs) for 1 h. All sections were washed three times with PBSF, 5 min each between antibody incubations. The color reaction was developed by adding 0.05% 3′-3′-diaminobenzidine tetrahydrochloride and 0.003% H₂O₂ in Tris buffer to reveal the peroxidase activity. All sections were counterstained with methyl green for tissue profile.

BrdU-positive cells were manually counted in every section in areas of the neural tube, heart, lungs, and neural crest. A partial correction of overcounting between adjacent sections was made using the Abercrombie formula. The section thickness and penetration of immunostaining were considered.

Immunohistochemistry analysis of embryos

For cyclin D1 immunohistochemistry, paired embryos in gel were cut into 40 μm coronal sections with a Leica 1000S vibratome. The sections were then washed three times in PBS and incubated in PBS containing 0.3% Triton X-100 (TX) overnight at room temperature. Tissue was washed three times for 5 min each, and incubated in MOM per manufacturer’s directions (Immunodetection Kit, Vector Laboratories, Burlingame, CA) to reduce nonspecific binding. Tissues were washed, and then incubated in MOM working solution containing a 1/100 dilution of monoclonal cyclin D1 antibody (Cell Signaling Tech) for 24 h at room temperature. Tissues were rinsed three times with PBS and incubated with a goat antimouse Alexafluor 488 antibody (Molecular Probes) at a dilution of 1/200 for 3 h at room temperature. Tissue was rinsed three times with PBS and equilibrated in 2× SSC for PI staining.

Propidium iodide was used as a counterstain in all tissue used for cyclin D1 immunohistochemistry. Cells were incubated for 20 min in 2× SSC with 20 units DNase free RNase (Promega), washed in 2× SSC, and incubated for 30 min in a 1 mg/ml solution of propidium iodide, then washed again in 2× SSC. Tissues were then fixed on slides, dried, coverslipped using a 50% mineral oil to preserve fluorescence and then viewed by microscopy.

Densitometry analysis

The mean density measures for BrdU were obtained using NIH image. Paired embryos in gel were cut into 40-μm coronal sections with a Leica 1000S vibratome. BrdU labeling was measured for the entire tissue areas as described below. The neural crest region was defined as all tissue from the dorsal tip of the neural tube, lateral to the neural tube, back to the ventral edge of the first arch mesenchyme, and in a straight line to the ventral edge of the floor plate (see Figure 4A). Density was measured for the entire tissue averaged over four animals for each treatment group, with three different coronal serial sections for each animal. To normalize for equal area, measurements for mean density included a measure for area. The smallest area for each tissue region was used and total density measurements were adjusted to that total area measurement for all samples and treatment groups. Therefore, comparisons between groups were made by taking the average density for BrdU per equal area for each tissue. Statistical analysis performed a Student’s t-test with Statview software (SAS Institute, Inc., Cary, NC).

Fluorescent intensity analysis for D1 and PI from embryo tissues

After staining, fluorescent intensity was measured for the entire heart and neural tube using NIH image software. Intensity averages were for three animals for each treatment group with three different 40-mm coronal serial sections for each animal for each tissue region. To control background fluorescence, background was subtracted by measurements taken from each slide in an area with no tissue (gel only). To normalize for equal area, measurements were taken for intensity and total area. The lowest measure of area for each tissue region was used and total intensity measurements were adjusted to that total area measurement. All data are reported as percentage of intensity change compared to controls set at 100%. In statistical analyses, Student’s t-test was performed with the Statview software (SAS Institute, Inc., Cary, NC) with P < 0.05.

RESULTS

DRG-NC study

Alterations in cell growth and DNA fragmentation. The alcohol concentration profile for DRG-NC cultures over 48 h showed a biphasic decrease in alcohol concentration of approximately 50% over the first 24 h and 25% for 24–48 h for both 200 and 400 mg/dl alcohol concentrations (Figure 1A). Cells exposed to either alcohol concentration demonstrated approximately 40% decrease in cell numbers within 6 h and maintained this reduction throughout the 48 h culture period. The control cultures showed no significant change in cell number through 48 h (Figure 1C). Propidium iodide (PI) staining of DNA showed compact and focal nuclear staining in control cells throughout all cell cycle stages (Figure 1B, controls). After alcohol exposure, there was an increase in pycnotic focal staining with disruption of nuclei, there were signs of DNA fragmentation, the cells often appeared smaller in size than normal, and broken cytoplasmic membranes were observed (Figure 1B, alcohol). The population of cells with signs of DNA fragmentation was dose dependent, demonstrating a >2-fold increase of cells with fragmentation within 6 h, >3-fold increase after 12 h, and peaking at >3.5-fold at 24 h for 400 mg/dl alcohol. At 200 mg/dl, DNA fragmentation increased by 50% within 6 h, >2-fold after 12 h, and peaked at >3.5-fold after 48 h (Figure 1D). The reductions of total viable cells at 200 and 400 mg/dl were similar although a dose-dependent increase in apoptosis was seen at 200 mg/dl. One possible reason for these results is that some of the DRG-NC at 400 mg/dl alcohol show an increase in proliferation, possibly as a compensatory mechanism to the increased cell losses.

Correlation of increased cyclin D1 and DNA fragmentation. Immunohistochemical analysis of cyclin D1 expression in DRG-NC cultures demonstrated notably three discernable
Figure 1. Alterations in DRG NSC growth and DNA fragmentation. DRG stem cells were exposed to 200 and 400 mg/dl alcohol and examined for DNA integrity and growth. (A) Alcohol concentration curve for DRG cell cultures over a 48 h period using 400 and 200 mg/dl. (B) Healthy control cells show compact DNA foci with PI staining (red). After alcohol exposure at either 200 or 400 mg/dl, cells show a considerable increase in DNA fragmentation (10 μm calibration). (C) Total cells in each culture were counted using Trypan exclusion, at 0, 6, 12, 24, and 48 h after alcohol treatment and are reported as a percentage of the starting cell count. A 40% decrease in cell numbers was seen within 12 h after alcohol treatment for either dose. (D) Cells with DNA fragmentation were counted using PI staining (red), and are reported as a percentage of total cell numbers. Significant damage was seen by 4 h. After 24 h of 400 mg/dl alcohol treatment, we found a >3.5-fold increase in apoptosis. *Denotes statistical significance between alcohol and control, **represents significant changes between control and other alcohol treatments. P < 0.05 Fisher’s Student’s t-test, N = 4.

Cellular expression phenotypes across the treatment groups—nonexpressing (undetectable), low D1 expression was defined as a light and diffuse cytoplasmic blue fluorescence), and high (D1, expressing cells showing bright fluorescence in both cytoplasmic and nuclear regions). The control DRG-NC showed that 34% of the total cell population had low cytoplasmic D1 protein expression (Figure 2B arrowhead, graph), and 3% demonstrated high cyclin D1 levels representing cells in late G1, (Figure 2A, arrowhead). Consistent with previous cell culture studies (Hamel and Hanley-Hyde, 1997; Li et al., 2001), cyclin D1 protein expression was not detected in >50% of the cell population. Alcohol exposure induced a dose-dependent increase in high cyclin D1 expressing cells. Examining a ratio of high D1 to total cell counts, 200 mg/dl alcohol treatment showed a >2-fold increase and 400 mg/dl demonstrated approximately 5-fold increase, both within 12 h (Figure 2 graph).

Alcohol treatment caused a reduction in PI staining: approximately twice the number of cells in the alcohol-treated cultures as compared to controls showed (low) PI staining, which is defined by a DNA staining less than half of normal 2N content, with pycnotic foci, and low nuclear PI staining (see Figure 2C, D arrows). Many cells with low-level PI staining also demonstrated (high) D1 expression (Figure 2C). Double staining of PI and D1 indicated that alcohol exposure also induced a cell population with increased D1 expression accompanied by elevated PI staining, defined as high-PI-expressing pycnosis (Figure 2D arrowhead); most of these cells also demonstrated fragmented DNA.
Fig 2. Analysis of cyclin D1 expression and DNA integrity in DRG-NSC cultures. Cultured DRG-NSC (control or 400 mg/dl alcohol for 12 h) were labeled by immunohistochemistry for cyclin D1 (blue) then counterstained with propidium iodide (red). Low D1 expression was defined as a light and diffuse cytoplasmic blue fluorescence. High D1 expression was seen as bright blue focal fluorescence in both cytoplasmic and nuclear regions. Low PI staining was defined as pycnotic cells with little, compact DNA. High PI staining was represented by disrupted DNA foci with >2-fold normal DNA content. Cells typical of control cultures are seen in (A, B). (A) Shows high D1 with low PI content representative of late G1 phase (arrowhead), or high PI with low D1 (arrow) representing S/G2 phase. (B) Shows low PI with low D1 label representative of mitosis (arrows and B arrowhead). Many cells have an increase in DNA fragmentation (C and D arrows) with an accompanying high cyclin D1 expression (arrowhead) after alcohol exposure, suggesting an apoptotic cell population with a high G1 marker. Labeled cells from control cultures, or those exposed to 200 or 400 mg/dl for 12 h, were counted for either high or low expression of cyclin D1 and DNA content. Graph demonstrates a dose-dependent alcohol-induced increase in cyclin D1 with 400 mg/dl alcohol, suggesting an increased population of cells in G1 or early S-phase. In statistical analyses, Fisher’s Student’s t-test was performed for N = 3. * Denotes significance between control and alcohol treatment. ** Denotes significance between control and alcohol groups, P < 0.03. Scale (A, C) 10 µm; (B, D) 5 µm.

Whole embryo culture study

G1/S-phase cell cycle protein expression. Whole embryo Western blot analysis demonstrated a >4-fold increase in cyclin D1 expression, and an accompanying 20% increase in cyclin D2. Examination of protein expression of the downstream transcription factor E2F1 showed a 25% increase in protein after alcohol exposure (Figure 3). The alcohol-treated embryos also
Figure 3. Western blot analysis of embryonic cell cycle proteins. Both alcohol and control protein (40 µg) samples were run on SDS–PAGE gels and detected by chemical luminescence. GAPDH was used as an internal control. Statistical analysis of band density by Student’s t-test showed that alcohol increases protein expression of E2F1, cyclin D1, cyclin D2, p53, and phosphorylated p53 (P ≤ 0.005).

$demonstrated a 22% increase in p53 protein (Figure 3), and a 24% increase in phosphorylated p53 (ser20).

Cellular proliferation in the developing organs. Two-hour BrdU incubation resulted in a reduction in the number of BrdU+ cells found in the neural tube, lung buds, neural crest cells (including facial, facio-acoustic, and trigeminal), the developing trachea, and the atrium of the heart (Figures 4A, B and 5E, F and G, H). Alcohol treatment also caused a reduced density of BrdU+ cells in the neural tube, neural crest, and heart atrium as compared to the controls (Figure 4B). In addition, the size of the developing organs with differential BrdU labeling was also altered by alcohol treatment, as indicated in serial sections throughout the embryo (Figure 4C). We observed a reduction in cell numbers for neural crest cells in the regions of facial, facio-acoustic, and trigeminal areas. In contrast, the mesenchyme region lateral to the neural tube (neural crest region) increased in size (Figures 4C and 5A and B) with an observed loss of cell numbers and tissue cohesiveness within this area (Figure 5A, B). Furthermore, alcohol exposure induced an enlargement of the ventricle of the neural tube (Figures 4C, 5A, B), while the area of the neural tube tissue was unchanged. In control embryos, BrdU+ cells were found throughout the neural tube tissue with a greater density lining the ventricle wall (Figure 5C). After alcohol exposure, there were overall reductions in BrdU+ cells in the neural tube, specifically reductions in cells lining the ventricle wall (Figure 5D arrows).

Alterations in DNA content and cyclin D1 expression during organ development. In the caudal neural tube, alcohol treatment increased cyclin D1 expression in the paraxial mesenchyme somites (Figure 6: 2), and both floor and roof plates of the developing spinal cord (6: 2′, and 6b, respectively). PI staining showed increases in the somites (6: 2), tissue ventral to the notochord (Figure 6: 2′), and along the lateral walls (Figure 6: 2′) and floor plate of the developing spinal cord.

In the rostral neural tube, alcohol treatment increased PI staining in the floor plate (Figures 6: 4′) and lateral walls (Figure 6, compare 4, 4′) (Figures 6, 8A, B). Noticeably, both PI and D1 staining in the untreated control neural tubes were concentrated in regions immediately adjacent to the lumen, and those exposed to alcohol showed a less confined pattern of expression. Fluorescent intensity analysis showed higher PI staining in the entire neural tube in the alcohol treatment group compared to control (Figure 7).
Figure 4. Proliferation analysis by BrdU incorporation in dividing cells (controls, solid black; alcohol exposed, solid gray). (A) Alcohol reduces the amount of BrdU incorporation indicating S-phase cell cycle alterations during cell division. The reductions were evident in the neural tube, neural crest, lung buds, and atrium. (B) Mean density measures/equal area supports reduced proliferative cell counts from A. (C) Total area measures show marked reductions in neural crest cells, with increases in the surrounding head mesenchyme tissue (neural crest region), and the ventricle of the neural tube. In statistical analysis, Student’s t-test was performed for $P < 0.01$, $N = 4$ independent animals for each group. Scale bars—A, B, D, E: 100 $\mu$m; C, D: 10 $\mu$m; E, F: 40 $\mu$m.

In the limb buds, alcohol exposure reduced PI staining and the expression of cyclin D1, and altered their patterns of expression. In controls, D1 expression and PI staining were evenly dispersed throughout the limb bud, with some focal D1 expression seen in vasculature. Alcohol exposure reduced D1 expression and PI staining overall, and induced increases in overlapping expression for both D1 and PI staining within the vasculature (Figure 6A, labels 1 and 1′

In the heart, alcohol induced alterations both in PI and D1 staining and considerable morphological changes. The D1 and PI fluorescent intensity increased approximately 20% and 15%, respectively, in the total heart tissue (Figures 6 and 7), particularly in areas of the atrium and ventricle walls and septums (Figure 6: 3, 3′′ and 3′, respectively). These areas, and areas of cardiac gel and outflow tract, also showed a substantial increase in DNA fragmentation (Figure 8C: C′ and D, D′, D′′), as indicated by PI staining.

A particularly notable outcome is that alcohol treatment caused an ectopic distribution of D1 and PI staining in hematopoietic cells in the limb buds, heart, and caudal neural tube (6a: 1, 1′, 2′′, 3′). In all cases there were focal regions of cells with increased D1 expression, and in the cases of the limb bud and heart, a correlative increase occurred in PI staining of these same cells.

DISCUSSION

The hallmarks of growth retardation and microencephaly in FASD are likely attributes of defects in gene expression, cell proliferation, and apoptosis (Goodlett et al., 2005; Sulik, 2005), but the mechanism(s) underlying these changes are unclear. To investigate how alterations in specific cell cycle proteins may affect proliferation and cell losses, we examined alcohol-induced changes in proliferation, protein expression of early cell cycle regulators that affect G1/S-phase progression, and apoptosis. Experiments using DRG-NC showed reduced proliferation and a correlated increase in apoptosis. Further, using DRG-NC and embryo cultures, we provide data indicating that alterations in G1 cyclin expression and E2F1 are associated with reduced proliferation, induction of apoptosis via DNA damage, and increased cell losses. These expressional changes take place during early fetal developments and differentially affect several organs and tissue types. We propose that aberrant
expression of G1 cyclins and E2F1 contributes to altered cell cycle progression and apoptotic induction, and represents one mechanism that contributes to growth retardation and cell losses from fetal alcohol exposure.

Previous studies with cultured cerebellar granule progenitor cells and with postnatal cerebellum rat neonates demonstrated that changes in cell cycle regulation at the G1/S-phase transition may occur upon alcohol exposure (Huang et al., 2001; Li, Miller and Luo, 2002). Alterations were seen in cyclin D2/CDK6 and cyclin A/CDK2 expression as well as CDK inhibitory proteins all associated with regulation of the G1/S-phase transition. Some more studies examining alcohol-induced cell cycle progression have demonstrated slowed G1 and S-phase progression after alcohol exposure (Luo and Miller, 1998; Luo et al., 1999; Siegenthaler and Miller, 2005).

Our current Western analysis data of whole embryo cultures demonstrated that alcohol increased the expression of proteins including cyclin D1, D2, and E2F1, which are key players in G1/S-phase cell cycle regulation and subsequent proliferation. In addition, alcohol exposure to DRG-NC altered the expression of cyclin D1 and cells overexpressing cyclin D1 often demonstrated DNA fragmentation. It has been suggested that high levels of cyclin D1 or E2F1 may activate apoptosis through p53 phosphorylation and inhibition of p53 degradation (Phillips et al., 1997). Our protein expression data demonstrated an increase in both p53 and serine 20 phosphorylated p53. In this

Figure 5. Comparison of BrdU incorporation for regions of the developing embryo. Controls (A, C, E, G) versus alcohol-treated (B, D, F, H). Alcohol induced decreases in BrdU incorporation in the neural tube (A, B, C, D), heart atrium and ventricle (G, H), and lung bud and trachea (E, F). Alcohol also induced increases in the size of the heart ventricle (G, H), and neural tube ventricle.” Denotes the neural tube ventricle in (A), and altered structural integrity of the mesenchyme of the neural crest including neural crest cell populations (A, B labeled). Arrows depict regions of distinct change in BrdU incorporation. Distinct alterations in BrdU-labeled cells were seen in the neural tube, with alcohol-induced proliferation of cells away from the ventricle walls, often in clusters (D arrow heads). V, neural tube ventricle. Scale markers—A, B, G, H: 100 µm; E, F: 40 µm; C, D: 10 µm.
Figure 6. Fluorescent micrographs for cyclin D1 (green) and propidium iodide (red) from areas of control and alcohol exposed E8.25 + 2 embryos. Arrows depict regions with distinct alterations in DNA content, and correlations to altered cyclin D1. In the limb buds, alcohol-induced decreases in overall cyclin D1 and DNA, with increases in developing vasculature (A, 1, 1'). In the caudal regions of the neural tube, alcohol-induced overlapping increases in cyclin D1 and DNA content in the floor plate (2'), areas of developing somite mesenchyme (2) and hematopoietic cells ventral to the notochord (2''). A distinct region in the neural tube roof plate shows a cell population with high D1 staining, suggesting a synchronized proliferation (B). In the developing heart overall decreases are seen in D1 and DNA. However, several overlapping increases in both D1 and DNA are seen in the atria (3,3'') and ventricular walls (3'). Areas of the rostral neural tube, including the floor plate and lateral walls, show an overlapping increase in expression of both markers (4', 4, and 4', respectively) from alcohol. DNA is increased throughout the neural tube, with no significant change in overall D1. Scale bars: limb buds 25 µm, all others 100 µm.

regard, it will be of interest to investigate whether the Chk1 and Chk2 kinases, known to phosphorylate p53 downstream of the ataxia telangiectasia-mutated gene (ATM) (Powers et al., 2004; Rogoff and Kowalik, 2004), might be responsible for phosphorylation of serine-20 on p53 (Enoch and Norbury, 1995). Thus the E2F1–ATM–p53 pathway is one possible mechanism that connects cell cycle misregulation to the apoptotic cell losses as a consequence of alcohol treatment.

Alterations of protein expression associated with cell cycle regulation and apoptosis

Our initial experiments using DRG-NC cultures demonstrated an alcohol-induced decrease in proliferation within 6 h of alcohol exposure and a correlative increase in apoptotic cell numbers within the same timeframe. We demonstrated an increase in cells with both elevated D1 expression and nuclear pycnosis in these neural stem cells. This suggests that by misregulating cyclin D1, alcohol is able to alter proliferation (particularly in G1 or S-phase cell cycle progression) and induce apoptosis. To determine the system-wide alteration of cell cycle progression, we used whole embryo cultures to examine changes in DNA synthesis and altered G1/S-phase progression in embryo tissue. BrdU labeling in cultured embryos showed a decrease in BrdU+ cell numbers in several developing organs. Distinct decreases were seen in the developing neural crest, lung buds, neural tube, and heart atrium. Interestingly, this reduced DNA synthesis correlated well with tissues that showed alterations...
in morphology during this period of development. From protein expression data and our results from BrdU labeling, we hypothesize that a population of cells from the developing embryo enters S-phase prematurely, is incapable of completing DNA replication, and is likely staged for apoptosis via a p53 DNA damage mechanism. In light of current data, it will be interesting to examine components of the ATM/CHK1/2/p53 DNA damage pathway for alterations in kinase activity directed toward p53.

Distinct alterations in proliferation, cell cycle proteins, and DNA fragmentation in the heart and neural tube

Previous studies using chick embryos suggested an increased apoptotic sensitivity of neural crest cells to alcohol during early development that contributes to dysmorphology (Cartwright and Smith, 1995). Our data demonstrate distinct alcohol-induced changes in proliferation, cyclin D1 expression, and DNA content, and further demonstrates a differential tissue-specific cellular response, the most prominent examples being within the neural tube and heart tissues. In control neural tubes, a 2-h BrdU-labeling showed an overall reduced cell proliferation. Expression of cyclin D1 was unchanged between treatment groups with no evidence of expression pattern changes. However, in opposition to BrdU incorporation, alcohol induced increases in DNA content throughout the neural tube, most prominently within the floor plate and lateral walls, with no evidence of increased DNA fragmentation or apoptosis. It is possible that the increased DNA content and reduced proliferation represent an increased cell population with slowed (stalled) cell cycle progression in S or G2 phase. This is supported by previous studies using cerebellar granule progenitors, which showed ethanol-induced cell cycle alterations, with increases in the duration of S-phase (Miller and Nowakowski, 1991; Miller and Kuhn, 1995; Luo et al., 1999; Li et al., 2001).

However, in contrast to the neural tube, alcohol-exposed embryos show a distinctly different set of alterations in DNA content and cyclin D1 expression in specific regions of the heart. Alcohol exposure increased the expression of cyclin D1 in the walls of the atrium, ventricles, and septums of the heart. This correlated with increases in DNA content in the same tissue structures, with many of these cells showing substantial increases in DNA fragmentation. Tissue integrity within these developing chamber walls and septum regions were also disrupted, and correlated with cell populations showing D1 increases and DNA fragmentation. BrdU-labeling showed a reduced proliferation in areas of the atrium, with no significant changes seen in other areas within the heart. These data correlate well with results from DRG-NC cultures, with reduced proliferation, increases in cells with elevated cyclin D1 and DNA content, and increased DNA fragmentation. This suggested that alcohol induced alterations in the G1/S phase transition, disrupted DNA synthesis, and induced apoptosis. Dissecting the mechanism(s) that induce such responses will be important in understanding the role that cell cycle misregulation plays in altering tissue morphology associated with

Figure 7. Histograms for fluorescent intensity measurements from the heart and neural tube for both cyclin D1 and propidium iodide. *Denotes those giving statistically significant values in a Fisher’s Student’s t-test (P < 0.03). Three serial sections were used from three independent animals.
FASD. Equally important is the fact that we will be able to understand how specific cell populations respond differently to alcohol, and how distinct developmental stages may alter tissue sensitive responses. Interestingly, several distinct populations of developing hematopoietic cells, including areas in the heart, limb buds, and the developing gut, demonstrated reduced BrdU-labeling, with increased DNA content and cyclin D1 expression. In all cases, there were distinct reductions in cell numbers. This represented a unique cell population with alcohol-induced alterations in cell cycle and DNA alterations similar to those seen in DRG-NC and heart tissue.

In summary, we demonstrated that alcohol exposure to DRG-NC and whole embryonic cultures increased early cell cycle proteins that are responsible for proper G1/S phase regulation, and altered DNA synthesis. The misregulated cyclin D1, and E2F1 in the cell cycle, and p53 in the apoptotic pathway, are likely key components in the induction of DNA damage and apoptosis seen in specific embryo tissue. Furthermore, a common, as well as distinct, pattern of cell cycle alternation and apoptotic induction were observed in different organs (e.g., heart/hematopoietic tissue vs. neural tube) indicating an alteration in cell cycle progression through multiple mechanism(s).

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