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Essential Role of Extracellular Matrix (ECM) Overlay in Establishing the Functional Integrity of Primary Neonatal Rat Sertoli Cell/Gonocyte Co-cultures: An Improved *In Vitro* Model for Assessment of Male Reproductive Toxicity

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The development of *in vitro* models for testicular toxicity may provide important tools for investigating specific mechanisms of toxicity in the testis. Although various systems have been reported, their application in toxicological studies has been limited by the poor ability to replicate the complex biochemical, molecular, and functional interactions observed in the testis. In the present study, we evaluated a significantly improved Sertoli cell/gonocyte co-culture (SGC) system that employs a 3-dimensional extracellular matrix Matrigel (ECM) applied as an overlay instead of a substratum. We explored the dose- and time-dependent effects of the addition of such an ECM overlay on cytoskeletal and morphological changes in the SGC system, and the resulting effects on cellular integrity. Furthermore, we correlated the latter effects with the ECM-dependent modulation of stress and survival signaling pathways and, most critically, the expression levels of the spermatogonia-specific protein, c-Kit. Finally, we applied this co-culture system to investigate the dose- and time-dependent effects on the morphology and induction of apoptosis of cadmium. We observed that the dose-dependent addition of an ECM overlay led to an enhanced attachment of Sertoli cells and facilitated the establishment of SGC communication and cytoskeletal structure, with a dramatic improvement in cell viability. The latter was consistent with the observed dose- and time-dependent modulation of both stress signaling pathways (SAPK/JNK) and survival signaling pathways (ERK and AKT) in the presence of the ECM overlay. Furthermore, the dose-dependent stabilization of c-Kit protein expression confirmed the functional integrity of this co-culture system. We conclude that this modified SGC system will provide investigators with a simple, efficient, and highly reproducible alternative in the screen for testicular cell-specific cytotoxicity and the assessment of molecular mechanisms associated with both normal development and reproductive toxicity induced by environmental toxicants.

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INTRODUCTION

Reproductive disorders and hazards to reproductive health are a prominent public health issue (Swan, 2003; Dunson *et al.*, 2004). Widely cited associations of environmental exposure and the decline in sperm count or reproductive function strongly suggest the need to understand the extended mechanisms by which environmental agents reduce sperm numbers. *In vivo* testicular toxicity model systems have played important roles in elucidating the risk assessment of many testicular toxicants. Recent increasing restrictions on animal experiments have persuaded researchers to seek *in vitro* model alternatives that could reduce the use of experimental animals (Stokes *et al.*, 2002). The development of *in vitro* models also has the potential to provide unique methodology in the investigation of the specific mechanisms of toxicant action in the testis, leading to improvements in the interpretation of data obtained from *in vivo* systems. To date, various *in vitro* systems have been reported in the literature, including Sertoli cell/germ cell co-cultures (Hadley *et al.*, 1985; Gray, 1986), Sertoli cell-enriched cultures (Chapin et al., 1988), germ cell-enriched cultures (Lejeune et al., 1998), Leydig cell cultures (Yang et al., 2003), and Leydig-Sertoli cell co-cultures (Bilinska, 1989). Sertoli cell/germ cell co-cultures (SGC) have been used to examine the interactions and effects of various hormones and growth factors on spermatogonial survival and proliferation *in vitro* (Mather *et al.*, 1990). In addition, SGC isolated from fetal rat testes have been developed, and their interactions have been studied *in vitro* (Orth and Jester, 1995). However, limited applications in toxicological studies have been reported, specifically because of the lack of reproducibility of the cell isolation procedure (Gregotti *et al.*, 1992; Li *et al.*, 1998) and the poor ability of these *in vitro*

culture systems to replicate the complex biochemical, molecular, and functional interactions observed in the testis *in vivo*. Critical techniques employed in successful cell isolation include sequential enzymatic digestions and gravity sedimentation of Sertoli cell and germ cell clusters (Steinberger *et al.*, 1975; Welsh and Wiebe, 1975). The other key determinant for enhancing the stability of this *in vitro* technique is the use of a defined culture environment in which to replicate the *in vivo* architectural integrity. Recent improvements have focused on the use of an extracellular matrix coating, Matrigel, in tissue culture–treated dishes (2-D substratum) to enhance Sertoli cell attachment. Matrigel is referred to as *extracellular matrix* (ECM) in this article. It is a solubilized complex mixture extracted from a basement membrane protein–rich mouse tumor (Kleinman *et al.*, 1982); the major matrix components are laminin, collagen IV, entactin, and heparan sulfate proteoglycan. The matrix also contains growth factors, matrix metalloproteinases, and several undefined compounds. Although such ECM pre-coated dishes have been used with relative success in the culture of SGC (Hadley *et al.*, 1985; Orth *et al.*, 2000), the associated mechanism of improvement in cell survival and proliferation remains unclear. Furthermore, a recent report shows that 3-D matrix-mediated adhesions differ from 2-D matrix in focal and fibrillar adhesions, as characterized in their contents of $\alpha5\beta1$ and $\alpha v\beta3$ integrins, paxillin, and other cytoskeleton components (Cukierman *et al.*, 2001). These structural requirements have been shown to be critical in the maintenance of the 3-D architectural integrity needed to recreate the *in vivo*–like environment.

In this study, we propose that the employment of a 3-D culture environment, effected through the addition of an ECM overlay, will result in a significant improvement to the existing 2-D substratum approach traditionally used in the culture of SGC. We hypothesize that this modification provides a simpler and more efficient method to improve the interactions between Sertoli cells and germ cells via the reorganization of cellular architecture and the resultant modulation of both intracellular stress and survival signaling cascades, thereby leading to a physiologically stable SGC system.

To test our hypothesis, we explored the dose and time-dependent effects of ECM overlay on morphological and cytoskeleton changes, cell viability, and stress and survival signal transduction pathways. We also examined the expression levels of c-Kit, an important regulator in primordial germ cell survival and proliferation (Yoshinaga *et al.*, 1991). Finally, we applied this co-culture system to investigate the dose- and time-dependent effects of cadmium (Cd), a known testicular reproductive toxicant (IARC, 1976.) in both animals and humans.

We found that an ECM overlay dose-dependently enhanced the attachment of Sertoli cells and facilitated the establishment of Sertoli cell–gonocyte communication, as observed by refinements to cytoskeleton structure and enhanced cell viability. The latter was consistent with the observed dose- and time-dependent modulation in the presence of the ECM overlay of both stress signaling pathways (SAPK/JNK) and survival signaling pathways (ERK and AKT). Furthermore, the dose-dependent increase of c-Kit protein expression in co-culture suggested that the presence of an ECM overlay significantly improved the proliferation and survival of the spermatogonia. Finally, dose-dependent effects of Cd treatment on morphological alteration and induction of apoptosis were observed in the culture SGC. We conclude that this modified *in vitro* SGC system will provide investigators a simple, efficient, and highly reproducible alternative in the assessment of molecular mechanisms associated with both normal development and reproductive toxicity induced by environmental agents.

MATERIALS AND METHODS

Sertoli Cell/Gonocyte Co-culture. Male pups from Sprague–Dawley rats were obtained from Harlan (Harlan, Kent, WA) with birth assigned as day 0. Testes were dissected from 5-day-old pups and sequential enzymatic digestion of testicular tubules was performed, essentially according to a previously reported method (Hadley *et al.*, 1985; Orth *et al.*, 1998). Briefly, testes were decapsulated under a dissection microscope, and the seminiferous cords/tubules were pooled and digested in a solution of 0.1% collagenase (Worthington Biochemical Corporation, Lakewood, NJ), 0.1% hyaluronidase (Sigma, St. Louis, MO), and DNAse I (Sigma in calcium, magnesium free Eagle's balanced salt solution (CMF-EBSS) (Life Technologies Inc., Gaithersburg, MD) for 20 min at 37°C. After this incubation, the tubules were allowed to settle down on ice for 10 min and then were further digested twice with 0.1% collagenase and DNAse I for 20 min at 37°C. A final digestion was performed by the addition of trypsin-ethylene diamine tetraacetic acid (EDTA) for 5 min, yielding a single cell suspension that was subsequently filtered through a 100 μm nylon mesh (Tetko, Briarcliff Manor, NY). This resulting cell suspension contained primarily Sertoli cells and type A spermatogonia. Cells were re-suspended in hormone-free and serum-free Eagle's minimal essential medium (Life Technologies, Inc.) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 3 mM sodium lactate, 1% ITS+ premix (a culture supplement containing insulin, transferrin, selenium, linoleic acid, and bovine serum albumin; BD Biosciences, Bedford, MA), and cell number and viability were determined by trypan blue exclusion. Cells were plated in 35-mm tissue culture–treated dishes at 2.4×10^6 density in serum-free medium. An ECM overlay was applied directly to the medium 30 min after seeding at a final concentration of 0, 50, 100, 200, and 300 μg/ml medium (Sidhu *et al.*, 1993); cells were then placed into a 37°C atmosphere in a humidified chamber with 95%/5% air/CO_2.

Cell Harvest and Protein Determination. At the appropriate time points, cultured cells were rinsed twice with ice-cold phosphate-buffered saline (PBS). Cell lysis buffer (Cell Signaling Technology Inc, Beverly, MA) was added to each dish, and cells were scraped with a rubber policeman. Harvested cells were then sonicated at 40 W for 15 s. Resultant cell lysates were centrifuged at 16,000×g for 15 min at 4°C. Supernatant fractions were collected, and the concentration of protein was determined with a commercially available kit (Protein Assay kit, Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard. All samples were subsequently stored at −80°C until assayed.

Morphology and Viability. All cultures were viewed with a Nikon inverted microscope equipped with phase-contrast optics (Nikon, Tokyo, Japan) at intervals during culture to assess their general appearance.

Resultant images were captured and digitized with a Coolsnap Camera (Roper Scientific, Inc, Duluth, GA). The digitized image was processed with Photoshop 5.5 software. To determine the viability of the cultured cells after overlay of ECM or Cd treatment, a three-color fluorescence assay was applied to determine the number of live and dead cells. Live cells have intracellular esterases that convert the non-fluorescent, cell permeable calcein acetoxymethyl ester (calcein AM) to the intensely green fluorescent, calcein. Propidium iodide (PI), a live cell–impermeable dye, selectively stained nuclei of later apoptotic nucleus with increased membrane permeability, while Hoechst 33342 selectively stained total nuclei with blue fluorescence. The nuclei of live cells are evenly stained, whereas apoptotic cells are intensely and irregularly stained. Propidium iodide (1 mg/ml), Hoechst 33342 (1 mg/ml), and calcein AM (Sigma) were added directly to co-cultures and incubated at 37°C for 5 min. The stained co-cultures were viewed with appropriate bandpass filters for PI, Hoechst, and calcein with Nikon Labophot-2 with E-Planachromats for brightfield and epi-fluorescence microscopes (Nikon, Tokyo, Japan). Each single-colored image was captured and digitized with a Spot Camera (Diagnostic Instruments, Sterling Heights, MI) equipped with MetaMorph software (Universal Imaging Corporation, Downingtown, PA), as shown later in Figure 2. Three random views were recorded and the number of the PI-positive cells (dead cells) and the number of nuclei stained with Hoechst (total cell number) were counted with the Cell Counting program in the MetaMorph software. Nuclei with intense and irregular dark-blue staining and without surrounding green were also counted (dying cells). The percentage of apoptotic cells was calculated by dividing the total number of dead (PI positive) and dying cells by the total cell number.

Visualization of the F-Actin Cytoskeleton. Co-cultures with different concentrations of ECM were washed with PBS 48 h after the addition of ECM overlay and fixed with methanol for 15 min at −20 °C, then washed twice with PBS and permeabilized in 0.1% Triton X-100 for 5 min. After permeabilization, cells were incubated in FITC-conjugated phalloidin (1 μg/ml, Sigma) for 40 min at room temperature. Vectashield mounting medium containing 2 μl Hoechst 33342 (1 mg/ml) was added to the dishes to counterstain nuclei. The stained co-cultures were viewed with an appropriate FITC filter under an epi-fluorescence microscope. The image was captured and digitized using a Spot Camera equipped with MetaMorph software.

Immunofluorescence of* c-Kit *Protein. Co-cultures of SGC were washed with phosphate buffered saline (PBS) 72 h after the addition of various concentrations of ECM overlay and fixed with 4% formaldehyde for 20 min, and then washed three times with PBS. After permeabilization in TBS containing 0.5% Triton X-100 (TX-100) for 10 min, cells were blocked for 30 min in 5% normal goat serum (NGS; Sigma) in PBS with 0.1% TX-100, and then incubated with primary c-Kit polyclonal antibody (1:200, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) in PBS with 1% goat serum and 0.1% TX-100 over night at 4°C. Cells were washed three times in PBS before the addition of a goat anti-rabbit Alexa-conjugated secondary antibody (1:200 dilution, Molecular Probes, Inc. Eugene, OR) for 1 h at 37°C. Vectashield mounting medium containing 2 μl Hoechst 33342 (1 mg/ml) was added to each dish to counterstain the nuclei and prevent photo bleaching. The stained co-cultures were viewed with an appropriate FITC filter under a fluorescent microscope. The image was captured and digitized with a Spot Camera equipped with MetaMorph software.

Cadmium (Cd) Treatments. Sertoli cell/germ cell co-cultures were cultured as described in the presence of an ECM overlay of 200 μg/ml. Serial dilutions were prepared from a stock solution of Cd and added directly at the stated concentrations to the culture medium 48 h after the addition of the ECM overlay. The final concentrations tested were 0.5, 2.5, 5, 10, and 20 μM. Morphological alterations to the co-culture after Cd treatment were recorded at the stated intervals during treatment. Similarly, the viability of the co-cultured cells after Cd treatment was assessed as described earlier under *Morphology and Viability.*

Assessment of Apoptosis. For the evaluation of the apoptotic morphological changes, cells were fixed and stained with Hoechst 33342 (0.1 mg/ml in PBS) after Cd treatment. The stained co-cultures were viewed with an appropriate filter under the fluorescent microscope. Each image was captured and digitized using Spot Camera (Diagnostic Instrument, Inc.) equipped with MetaMorph software. Apoptosis-associated endpoints were further determined in cell extracts by measuring functional activities associated with caspase 3/7 using caspase-specific fluorogenic substrates. The activity of caspase 3/7 was measured by a fluorometric assay, using Ac-DEVD-AMC as the specific substrate, as previously reported (Nicholson *et al.*, 1995; Shi *et al.*, 2000). Briefly, 10 μg of cell extract was added in duplicate in 96-well plate format. Reaction buffer containing the fluorogenic substrate enzyme-catalyzed release of 7-amino-4-methyl coumarin (AMC) was added to initiate the reaction, which was incubated at 37°C for 2 h and enzyme-catalyzed release of AMC measured by a fluorescence microplate reader at excitation 360 nm and emission 460 nm. Fluorescent units were converted to pmol of AMC released per microgram of protein and incubation time (h) using a standard curve generated with known serial dilutions of AMC.

Western Blot Analysis. Western blot analyses of the phosphorylation status of various proteins implicated in cellular stress and survival pathways were performed according to a method described in our previous studies (Sidhu *et al.*, 2004; Yu *et al.*, 2001). Gels were transferred to PVDF membranes (Millipore, Billerica, MA) using a vertical transfer apparatus (Bio-Rad Laboratories). Membranes were rinsed briefly in Tris-buffered saline, pH 7.6 (TBS), blocked with 5% nonfat dried milk in TBS with 0.1% Tween-20 (TTBS) for 20 min and rinsed again with TTBS. Membranes were then incubated overnight with primary antibody, followed by four washes with TTBS. The primary antibodies included c-Kit (Santa Cruz Biotechnology, Inc.), phospho-SAPK/JNK, phospho-ERK1/2, phospho-p38 MAPK, phospho-c-Jun, and phospho-ATF2 (Cell Signaling, Inc.). β-actin (Santa Cruz Biotechnology, Inc.) was used as an internal standard for the protein loading. No significant changes were observed in β-actin concentrations relative to cell number (protein concentration) under all our experimental conditions. After hybridization with relevant secondary antibodies conjugated to horseradish peroxidase, the resulting immunocomplex was detected with the ECL detection reagent (Amersham Biosciences Corp, Piscataway, NJ), followed by exposure to x-ray films. The resulting films were scanned using HP Scanjet 5400C scanner equipped with Precisionscan Pro 3.1 software, and the TIF image data were analyzed. Quantitative analysis of densitometric scans of band intensities and area were achieved using the "Quantity One" software (Bio-Rad Laboratories). Data are presented as arbitrary units after internal standard correction with β-actin.

Statistical Analysis. The results of quantitative analysis of cell number, percentage of apoptotic cells, caspase 3/7 activity, and Western blot band densitometric quantification are expressed as the mean ± S.D. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. A p value <0.05 denoted the presence of a statistically significant difference.

RESULTS

ECM Overlay Enhances Attachment and Overall Morphology of SGC in a Dose-Dependent Manner

To investigate the effects of ECM overlay on the morphology of SGC, primary Sertoli cells and gonocytes were isolated from neonate rat testis by sequential enzymatic digestion. The viability of the resultant cell suspensions was consistently >95%, with Sertoli cells being the predominant cell type

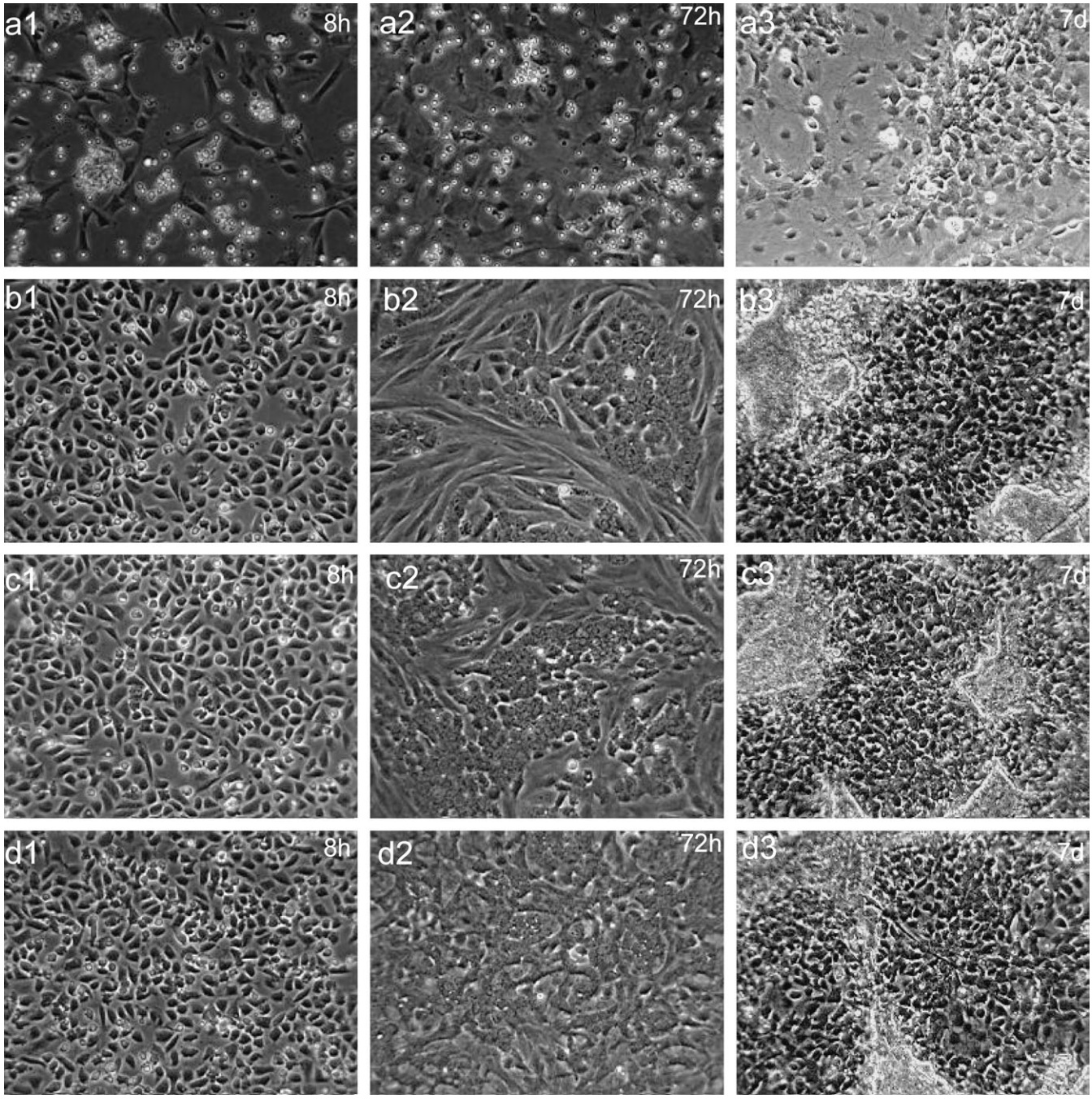
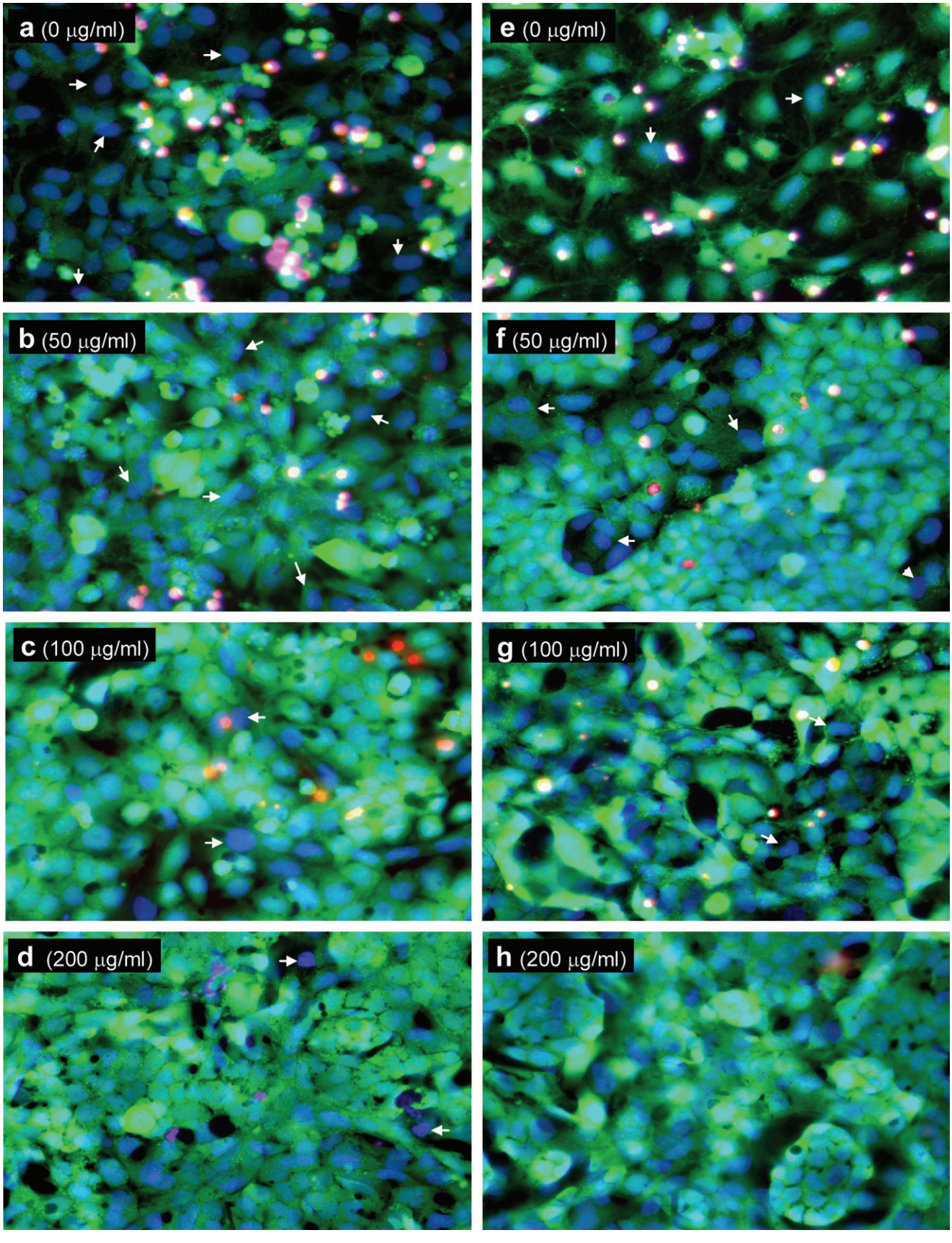


FIG. 1. Typical phase-contrast photography of neonatal Sertoli Cell/gonocyte co-cultures cultured in the absence or presence of an ECM overlay. Sertoli cells and gonocytes were isolated as stated in *Materials and Methods* and seeded at a density of 1.2×10^6 cells/ml. An overlay of ECM was immediately applied thereafter. The panels refer to the variable concentrations and duration of exposure to an ECM overlay: 0 μg/ml at 8 h (a1), 72 h (a2), and 7 days (a3); 50 μg/ml at 8 h (b1), 72 h (b2), and 7 days (b3); 100 μg/ml at 8 h (c1), 72 h (c2), and 7 days (c3); 200 μg/ml at 8 h (d1), 72 h (d2), and 7 days (d3). At least three replicates in three separate experiments were included.

and the remainder gonocytes. Cell attachment was observed to be poor (Fig. 1, a1–a3) and further diminished over the culture period without an ECM overlay. With the ECM overlay, however, Sertoli cells rapidly attached to the culture substratum, followed by deposition of gonocytes to the Sertoli cells. The gonocytes were easily distinguished by their larger cell size/morphology, and attached to the Sertoli cells ~3 h after the addition of the ECM overlay ($\geq$100 μg/ml). At ECM concentration of 50 and 100 μg/ml, initial attachment of Sertoli cells was enhanced, but subsequently resulted


a (0 μg/ml)
e (0 μg/ml)
b (50 μg/ml)
f (50 μg/ml)
c (100 μg/ml)
g (100 μg/ml)
d (200 μg/ml)
h (200 μg/ml)

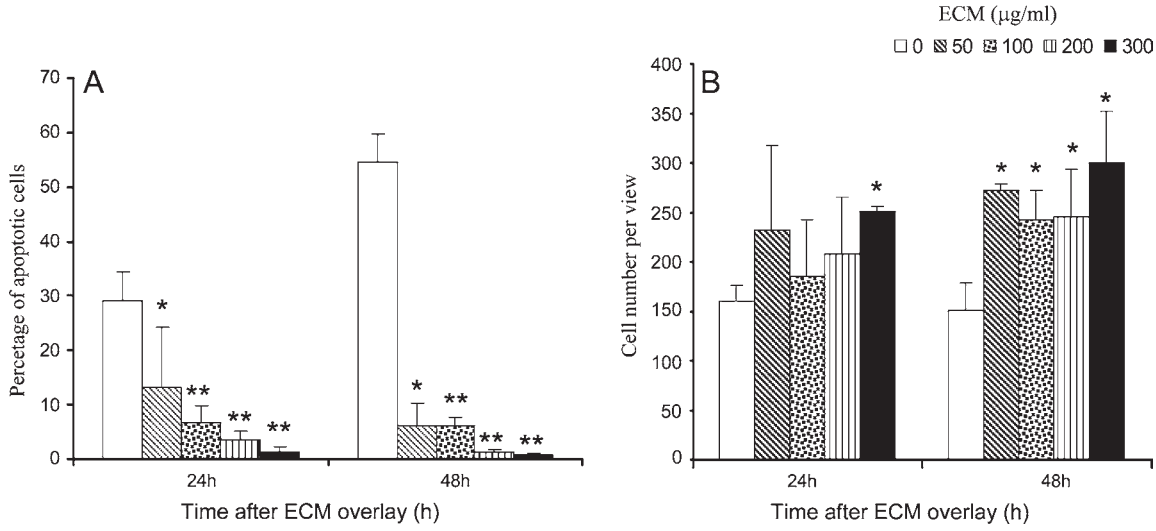


FIG. 3. Quantitative analysis of live/dead cells with the three-color fluorescence assay. Propidium iodide (PI), Hoechst 33342, and calcein AM were added directly to co-cultures and incubated at 37°C for 5 min, 24 h, or 48 h after the addition of an overlay of ECM. The stained co-cultures were viewed by epifluorescence with appropriate band pass filters for PI, Hoechst, and calcein. Each single-colored image was captured and digitized with a Spot camera equipped with MetaMorph software as shown in Figure 2. Three random views were recorded, and the number of dead cells (PI positive cells) and total number of nuclei, stained with Hoechst and calcein green, respectively (dying cells), and counted with the Cell Counting program of MetaMorph. The percentage of apoptotic cells was calculated by dividing the total number of dead and dying cells by the total cell number (panel A). The average number of cells per view is shown in panel B. Statistical significance was determined using Student's *t* test by comparing the 0 μg/ml group against the ECM addition group (* $p < 0.05$; ** $p < 0.01$). Three replicates in three separate experiments were included.

in increasing numbers of floating Sertoli cells and gonocytes by 8 h and 72 h (Fig. 1, b1–b2). The colonies of the SGC that remained attached were not evenly distributed on the dish after 72 h and 7 days. However, with an ECM concentration ≥200 μg/ml, a dramatic increase in Sertoli cell attachment was observed, and a multilayered structure formed 72 h post plating (Fig. 1, d2). No further effects were observed after the 72 h time-point, indicating a stabilization of cellular integrity after 72 h in culture. Seven days after seeding, a testicular-like multilayered architectural structure was observed as shown in Figure 1, d3. The modified SGC culture maintain its cellular structure integrity and viability at least for 2 weeks.

ECM Overlay Affects Cell Viability of SGC

To investigate the dose-dependent effect of the ECM overlay on the cell viability of the SGC co-culture, we employed a three-color fluorescence assay to measure the percentage of live/dead cells. Figure 2 shows the typical morphology of SGC with the different concentrations of ECM applied as an overlay and after 24 h (Fig. 2, a–d) and 48 h (Fig. 2, e–h) in culture. We observed a large number of PI-positive cells (dead cells) in co-cultures cultured in the absence of an ECM overlay at both 24 h (Fig. 2. a) and 48 h (Fig. 2, e), whereas there were few PI-positive cells in co-cultures where an ECM overlay (200 μg/ml) was applied whether these cells were cultured to 24 h (Fig. 2, d) or 48 h (Fig. 2, h). There were a number of PI-positive cells at lower concentrations (50 and 100 μg/ml) of ECM overlay after both 24 h (Fig. 2, b and c) and 48 h (Fig. 2, f and g). There were also a number of cells that lacked green calcein fluorescence (dying cells, arrows) in these ''suboptimal'' culture conditions. A multi-layer, 3-D architectural structure formed 48 h after the addition of ECM (≥200 μg/ml; Fig. 2, h).

With the MetaMorph software, we conducted a quantitative analysis of live/dead cells as assessed by the three-color fluorescence assay. The percentages of apoptotic cells and the average number of cells per view are shown in Figure 3. The percentage of apoptotic cells (condensed, irregular

FIG. 2. Typical high-magnification fluorescence-based photographs of live/dead cells assessed at 24 h (A) and 48 h (B). Propidium iodide (PI), Hoechst 33342 and calcein AM were added directly to co-cultures and incubated at 37°C for 5 min 24 or 48 h after the addition of an overlay of ECM. The stained co-cultures were viewed by epi-fluorescence with appropriate band pass filters for PI, Hoechst, and calcein. Each single-colored image was captured and digitized with a Spot camera equipped with MetaMorph software. The panels refer to the variable concentrations and duration of exposure to an ECM overlay: 0 μg/ml at 24 h (a) and 48 h (e); 50 μg/ml at 24 h (b) and 48 h (f); 100 μg/ml at 24 h (c) and 48 h (g); 200 μg/ml at 24 h (d) and 48 h (h). Arrows show the early apoptotic cells that exhibit intense blue staining without equivalent calcein green staining. Three replicates in three separate experiments were included.

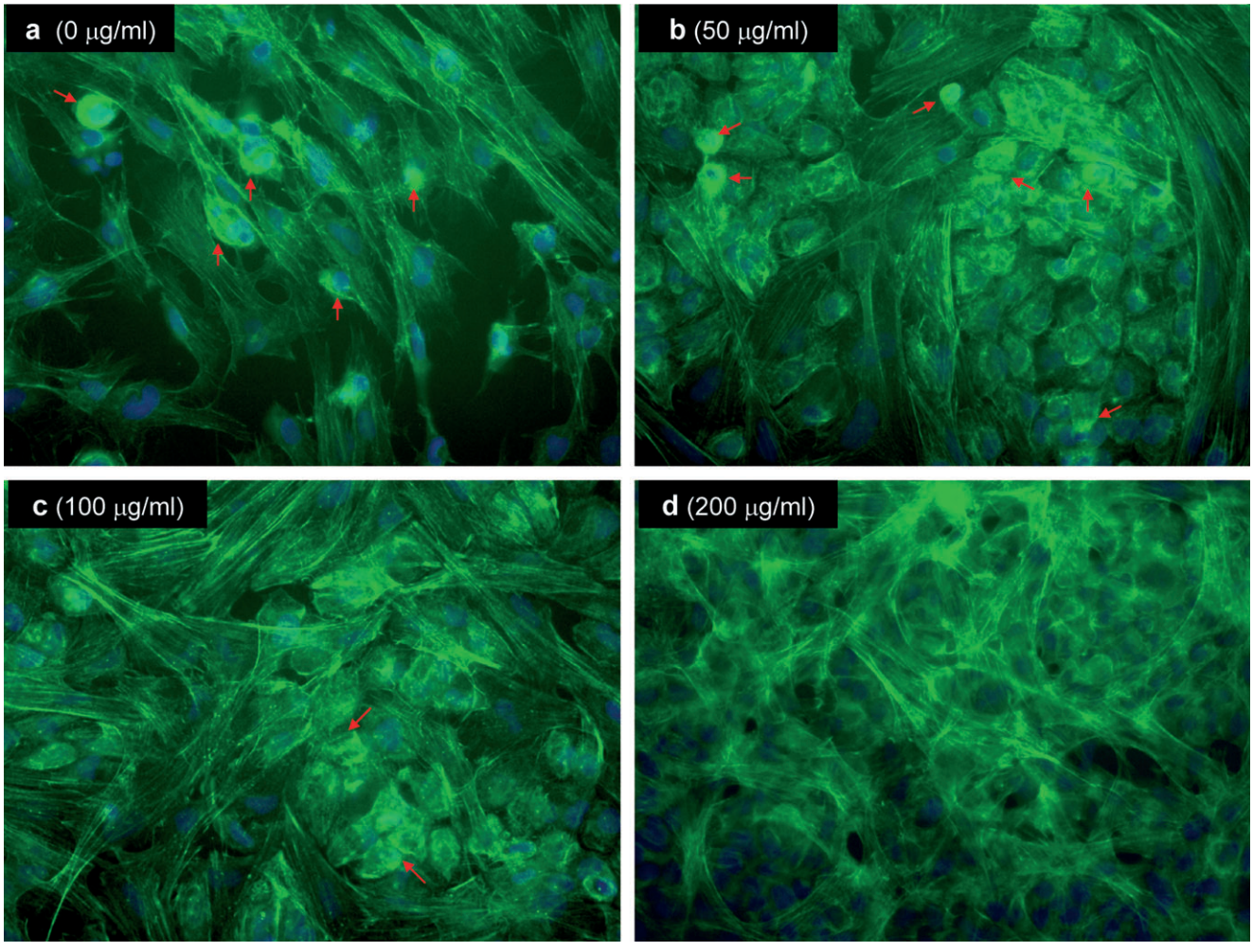


FIG. 4. Visualization of the F-actin cytoskeleton in Sertoli Cell/gonocyte co-cultures in the absence or presence of an ECM overlay: 0 (A), 50 (B), 100 (C), and 200 μg/ml (D). Sertoli cell/gonocyte cells were harvested 48 h after the addition of variable concentrations of ECM overlay and fixed with −20°C methanol for 15 minutes at −20°C. After permeabilization in 0.1% Triton X-100, cells were incubated in FITC-conjugated phalloidin (1μg/ml) for 40 min at room temperature. Vectashield mounting medium with 2 μl Hoechst 33342 (1 mg/ml) was added to each dish and covered with a coverslip to counterstain the nucleus and inhibit photobleaching. The stained co-cultures were viewed with an appropriate FITC filter under a fluorescent microscope. The image was captured and digitized with a Spot Camera equipped with MetaMorph software. Arrows show actin patches. Three replicates in three separate experiments were included.

staining with PI) decreased proportionally relative to the ECM concentration, both at 24 h and 48 h after plating, and was most significantly reduced when the concentration of ECM overlay was ≥200 μg/ml (Fig. 3A). The total number of cells was low in the co-culture without an ECM overlay, but it dose-dependently increased with an ECM overlay (Fig. 3B).

ECM Overlay Improves the Cytoskeletal (F-Actin) Structure of SGC

Sertoli cell–germ cell adhesions play a critical role in morphogenesis, establishment, and maintenance of tissue architecture, cell–cell communication, and normal cell growth and differentiation. The actin cytoskeleton and its associated proteins in these cells represent the organizers and motors for the dynamic shape changes observed in cell crawling, polarization, and cytokinesis (Huang and Ingber, 2000). To determine effects of ECM overlay in regulating the spatial and temporal dynamics between Sertoli cells, and Sertoli cell–gonocyte adhesion, we examined F-actin as a marker of cytoskeleton with the fluorescence probe phalloidin (Deerinck *et al.*, 1994). Co-cultures with different concentrations of ECM overlay were collected 48 h after ECM overlay and fixed with −20°C methanol and then stained with FITC-conjugated phalloidin. Figure 4 shows a typical micrograph associated with the fluorescence staining of F-actin. In the absence of an ECM overlay, cells at 48 h after plating were sparsely distributed and devoid of intimate cell–cell contacts. Long actin filaments could be found arranged in loose bundles in the flat, peripheral regions of the cytoplasm, and actin patches

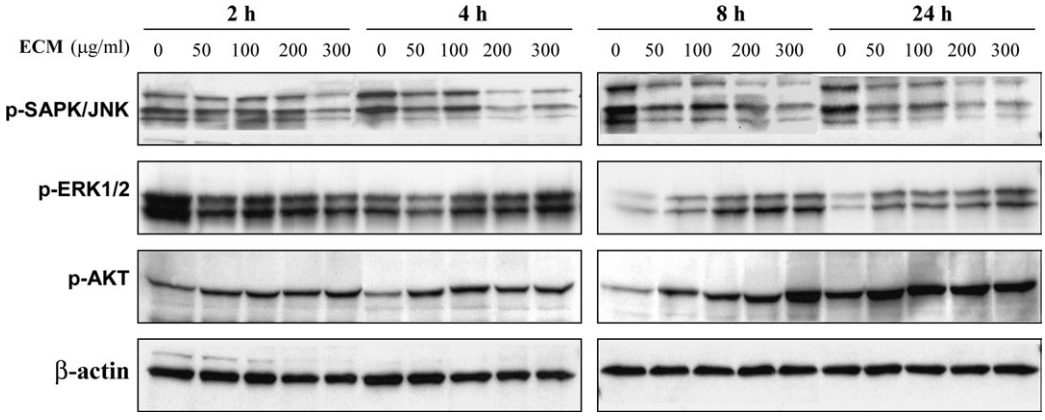


FIG. 5. Effect of ECM on stress (SAPK/JNK) and survival (ERK1/2, AKT) signal transduction pathways in Sertoli cell/gonocyte co-cultures. At various time points, co-cultured cells were harvested and cell extracts prepared and subjected to Western blot analysis of the phosphorylation status of SAPK/JNK, ERK1/2, AKT as described in *Materials and Methods.* β-actin was used as an internal standard for the protein loading. Gel is the representative of three replicates in three separate experiments.

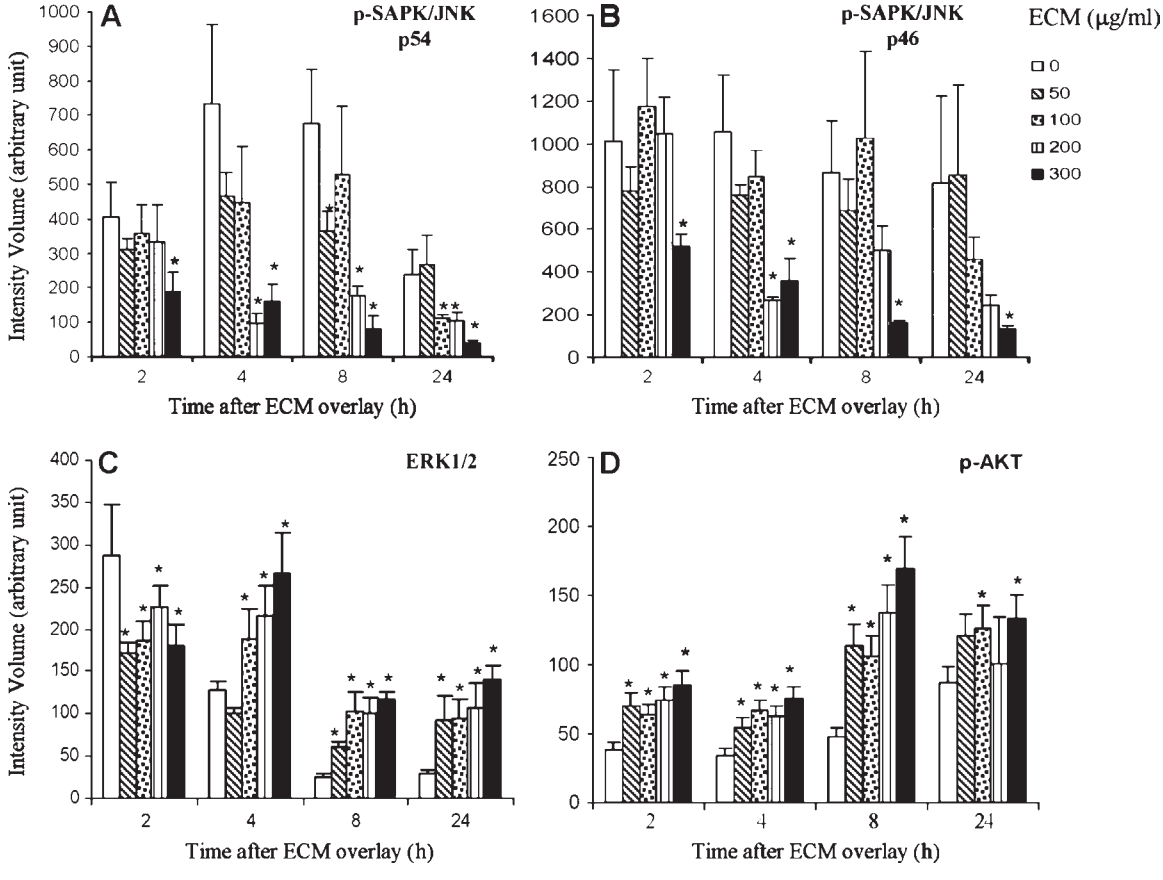


FIG. 6. Quantitative analysis of stress (SAPK/JNK) and survival (ERK1/2, AKT) signal transduction pathways in Sertoli cell/gonocyte co-cultures. Western-blot analyses were conducted as described in Figure 5. Quantification of resulting band intensities was achieved using the ''Quantity One'' software. Quantitative analysis of the phosphorylation of both bands associated with SAPK/JNK (p46/54) is shown in panels A and B; ERK1/2 in panel C; and AKT in panel D. Data are presented as arbitrary units after internal standard correction with β-actin. Each data point represents the mean ± SD of three separate experiments. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparison (* $p < 0.05$ and ** $p < 0.01$ as compared with the value of 0 μg/ml group at the same time point). Three replicates in three separate experiments were included.

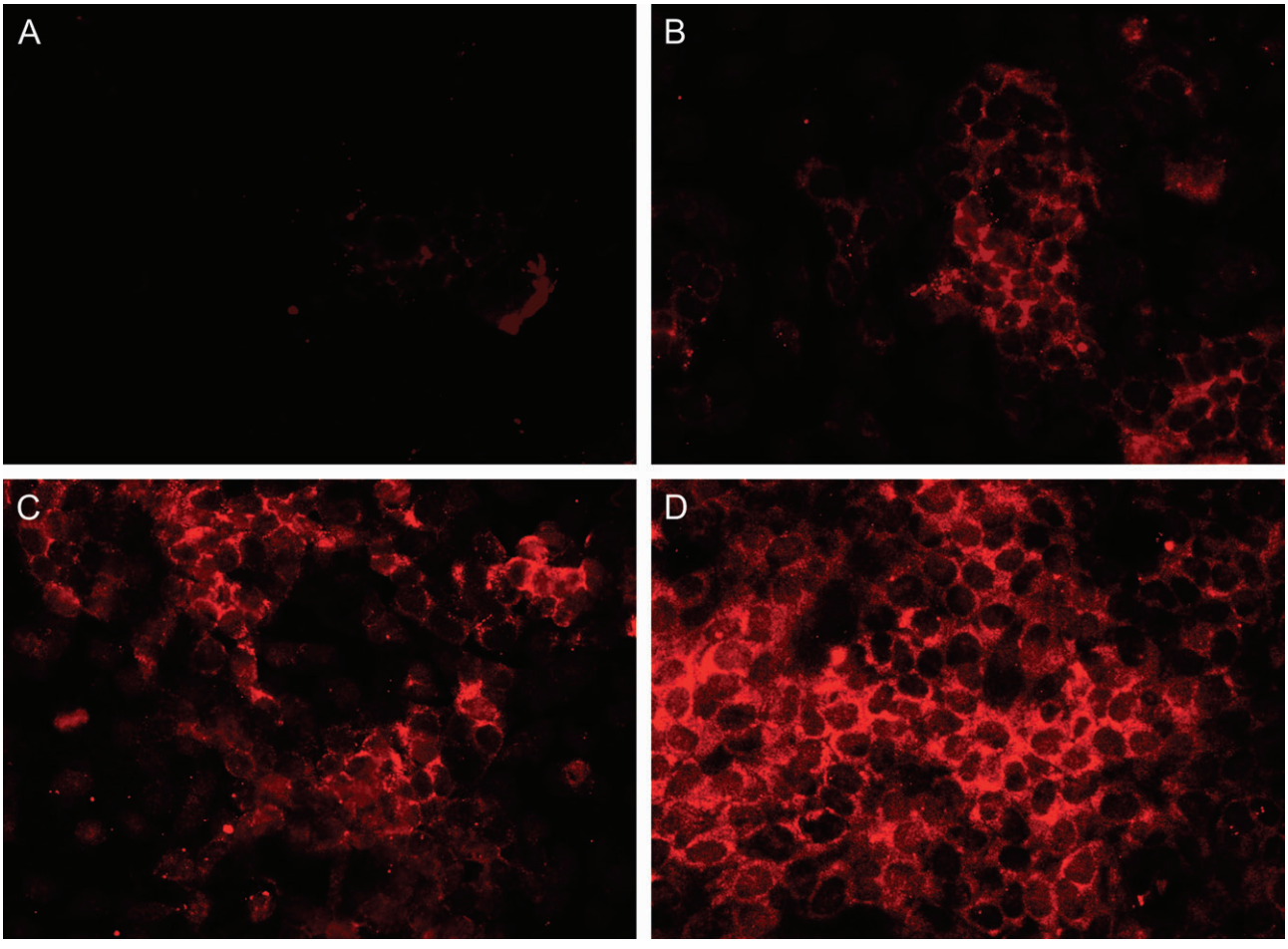


FIG. 7. Effect of ECM on the expression levels of c-Kit protein in Sertoli cell/gonocyte co-cultures. Sertoli Cell and gonocyte cells were harvested 48 h after the addition of variable concentrations of ECM overlay at 0 (A), 50 (B), 100 (C), and 200 (D) μg/ml and fixed with 10% formalin and subjected to immunofluorescence with a monoclonal antibody directed against c-Kit. For Western blot analysis, cells were harvested and subjected to Western blot analysis of c-Kit protein expression as described in *Materials and Methods*. A representative autoradiograph of three separate experiments is shown in panel E. Quantitative densitometric analysis of c-Kit was determined at the time points indicated. Data are presented as intensity volume in arbitrary units after internal standard correction with β-actin. Each data point represents the mean ± SD of three replicates in three separate experiments. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparison (* $p < 0.05$ and ** $p < 0.01$ as compared with the value of 0 μg/ml group).

were formed at the edges of the cells (Fig. 4A). In cells cultured with a lower ECM concentration, the cytoskeleton appeared to be less organized at sites of cell–cell contact, as demonstrated by long green actin filaments appearing in loose bundles (Figs. 4B and 4C). There were many actin patches observed near the edges of cells cultured in the presence of ECM concentrations between 50 and 100 μg/ml (Figs. 4B and 4C). At a concentration of ECM ≥200 μg/ml (Fig. 4D), FITC-phalloidin staining showed a diffuse cytoplasmic staining.

Modulation of Stress and Survival Signaling Pathways in SGC by ECM Overlay

The isolation of Sertoli cells and gonocytes from the testis involves multiple enzymatic digestions that can result in excessive cellular stress. Having demonstrated the significant improvements to cellular integrity effected in the presence of ECM overlay, we further examined whether these were associated with a parallel reduction of intracellular stress as determined by alterations to the phosphorylation status of key signaling proteins, notably members of mitogen-activated kinase family (MAPK) pathways. The MAPK are well-defined by their respective downstream proline-directed protein kinases including extracellular responses kinase (ERK1/2), stress-activated protein kinase/c-Jun amino-terminal kinase (SAPK/JNK), and p38 MAPK (Bhowmick *et al.*, 2001; Crean *et al.*, 2002; Rosen *et al.*, 2002). Utilizing a combination of Western analysis and phospho-specific antibodies, we examined the phosphorylation status of SAPK/JNK, ERK1/2, and p38 MAPK. As shown in Figures 5 and 6A and 6B, the presence of an ECM overlay resulted in a dramatic reduction in the phosphorylation of SAPK/JNK

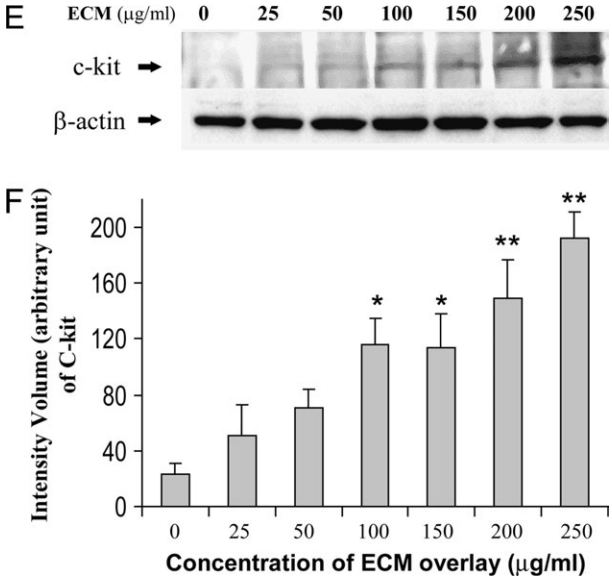


FIG. 7—*Continued.*

in a time- and dose-dependent manner (Figs. 5 and 6A and 6B). In fact, in the presence of an ECM overlay (300 μg/ml) a significant reduction in the SAPK/JNK stress signal was observed even at 2 h after plating. The SAPK/JNK stress signal was further reduced in the presence of ECM (200 and 300 μg/ml) at 4, 8, and 24 h after plating. The time-course and dose-dependent examination of ERK1/2 activation are shown in Figures 5 and 6C. In the absence of an ECM overlay, phosphorylation of ERK1/2 was markedly activated 2 h after plating, and then it decreased significantly at 8 h and 24 h (Fig. 6C). The ERK1/2 was depressed in a dose-dependent manner at the early time point post plating (2 h). However, the activation of ERK1/2 increased dose-dependently at the 8 h and 24 h time points. Furthermore, the activation of additional markers of stress, p38 MAPK, c-Jun, and ATF 2 was only observed in cells cultured in the complete absence of ECM overlay (data not shown), with only a faint band observed in the presence of ECM overlay culture, without any significant difference recorded between concentrations. These results provide supporting evidence for the important role of ECM in the regulation of stress and survival signaling pathway in the neonate Sertoli cells and gonocyte co-culture.

The serine/threonine kinase Akt, or protein kinase B (PKB), is a downstream effector of phosphatidylinositol 3 (PI 3)-kinase, and it has been shown to be the mediator of growth factor–dependent cell survival in a variety of cell types. Protein kinase B has also been shown to play a critical role in the regulation of testicular development (Datta *et al.*, 1999; Kandel and Hay, 1999). We postulated that the significant reduction in cellular stress exerted by the ECM would also be associated with parallel impacts on PKB/Akt-associated signaling. In a manner similar to that employed to study the impacts on cellular stress signaling, we examined activation of the Akt signaling pathway by Western-blot analysis coupled with phospho-specific antibody detection. Dose- and time–dependent activation of Akt was observed in the presence of ECM overlay (Figs. 5 and 6D). The activation of Akt was observed to increase significantly with time of co-culture from 2 h to 24 h at all treatments. An increase was also noted in a dose-dependent manner at the 2, 4, and 8 h time points; however, no significant difference was observed between the different concentrations of ECM overlay except without ECM overlay at 24 h.

Increased Expression of c-Kit Protein in SGC Cultured in the Presence of ECM Overlay

The *c-Kit* proto-oncogene encodes for a plasma membrane–associated receptor with inherent tyrosine kinase activity. We examined the levels of c-Kit protein expression in the SGC co-culture to determine whether the effect of ECM on the functional interaction between the Sertoli cells and gonocytes was associated with a modulation of this critical regulator protein. Figure 7 shows that an increase in c-Kit protein expression was observed in the presence of the ECM overlay, as detected by immunofluorescence staining and Western blot analysis 72 h after plating. We therefore conclude that ECM overlay did indeed facilitate an increased interaction between Sertoli cells and spermatogonia, and this forms a critical basis for the improvements in the cellular integrity as well as the *in vivo*–like environment.

Assessment of the Impact of Cd, a Reproductive Toxicant, in SGC

Finally, we applied this co-culture system to investigate the dose- and time-dependent effects of Cd treatment. The optimized concentration of ECM 200 μg/ml in SGC culture was used to examine the toxic effects of Cd treatment at concentrations ranging from 0.5 to 20 μM. The morphological observations demonstrate that Cd treatment at 10 and 20 μM significantly disrupted SGC structure at 8 h (Fig. 8B and 8C) and 24 h (Fig 8E and 8F), respectively, after treatment. Cell viability of the co-cultured cells after Cd treatment, as determined in a three-color fluorescence assay, also shows that apoptotic cells were clearly detected at a lower Cd concentration (5 μM), and dose-dependently increased (Fig. 9). In the morphological examination with the Hoechst nuclear staining, we consistently observed dose-dependent nuclear morphological changes, heavily condensed nuclei, nuclear shrinkage, and subsequent chromatin condensation into the periphery of the nuclei (Fig. 10 B–D). The caspase family of proteases plays a crucial role in apoptotic germ cell death. To identify the apoptotic mechanism involved in the Cd-induced cell death in the primary SGC co-cultures,

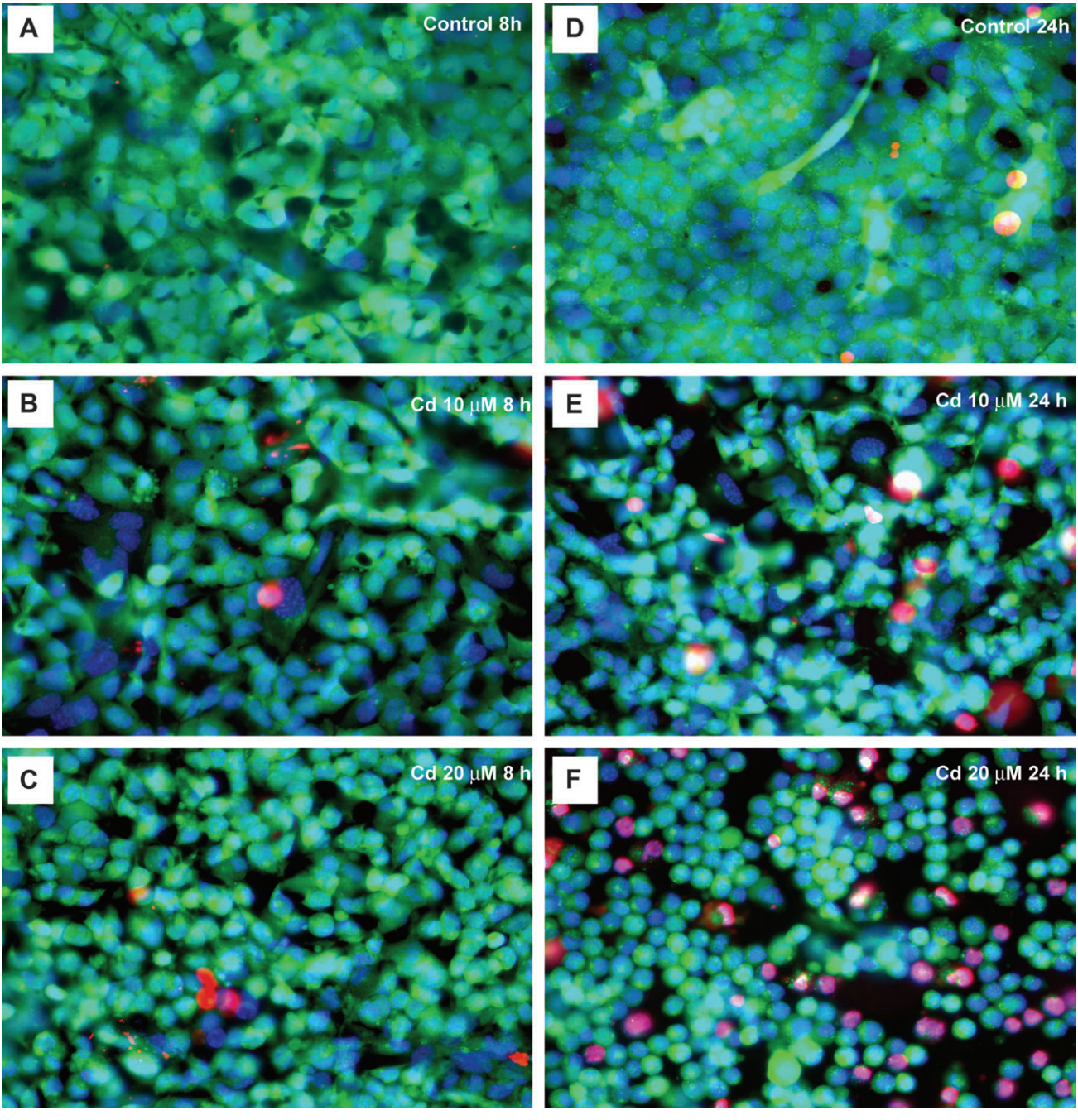


FIG. 8. Dose- and time-dependent effects of Cd on morphology (A–F) of Sertoli cell/gonocyte co-cultures. Propidium iodide (PI), Hoechst 33342, and calcein AM were added directly to co-cultures and incubated at 37°C for 5 min, 8 h or 24 h after Cd treatments. A three-color fluorescence assay was as described in Figure 2. Cd treatment caused dose-dependent morphological alterations indicative of loss of cell viability. Apoptotic cells (red PI positive cell) were observed to increase after 8 h at a concentration of 10 μM and then significantly at 24 h. Three replicates in three separate experiments were included.

we measured the caspase 3/7-like activity (Fig. 10E). At concentrations of 20 and 40 μM, Cd treatment induced majority cell death and significantly increased caspase-3/7–like activity 24 h after treatment. Furthermore, significant elevation of caspase 3/7 activity was observed at a lower concentration (5 μM), which is consistent with the morphological alteration and increase in the number and the percentage of apoptotic cells. These observations suggest that this modified co-culture system will prove to be a valuable tool for the fast testicular cell–specific screening for toxicity and

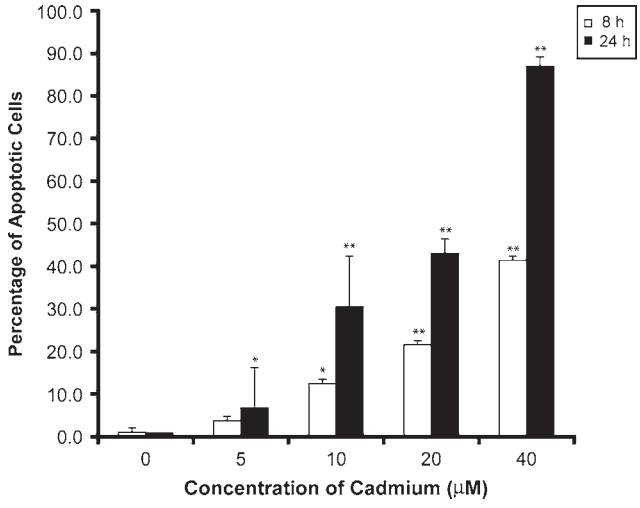


FIG. 9. Quantitative analysis of live/dead cells after Cd treatment with the three-color fluorescence assay. A three-color fluorescence assay was as described in Figure 2 and the quantitative analysis was as detailed in Figure 3. Statistical significance in the percentage of apoptotic cells was determined using Student's *t*-test by comparing the treatment against the control group (* $p < 0.05$; ** $p < 0.01$). Three replicates in three separate experiments were included.

for investigation of specific mechanisms of toxicity in the testis.

DISCUSSION

The development of *in vitro* model alternatives for testicular toxicity provides significant tools with which to investigate the molecular mechanisms associated with toxicant action in the testis. Various model systems have been reported in the literature; however, very limited applications in toxicological studies have been conducted using such systems. It has been difficult to stably culture Sertoli cell and germ cell co-cultures on artificial substrates such as plastic or glass (Gregotti *et al.*, 1992). Previous attempts to obtain more stable cultures have involved the use of an ECM substratum as the basal membrane in tissue culture dishes (2-D substratum), and although this approach has been successfully applied in establishing primary SGC (Hadley *et al.*, 1985; Orth *et al.*, 1998), its mechanism in regulating the interaction between Sertoli cells and germ cells is still unclear. In the present study, we modified the 2-D substrate method by applying ECM as an overlay (3-D matrix). To characterize this novel *in vitro* co-culture system, we examined the effects of ECM on morphology, viability, and survival; cytoskeleton structure, stress, and survival signal transduction pathways; and c-Kit protein expression.

In the intact organism, cells form tissues that are bound together by a meshwork of extracellular matrix (ECM) molecules that include collagens, fibronectin, laminin, and several other proteins (Semino *et al.*, 2003). Isolation of Sertoli cells and gonocytes involves a series of enzymatic digestions of the interstitial matrix and mechanical disruption of the tissue. Unfortunately, this procedure disrupts the interaction between the ECM and actin cytoskeleton, which is an essential microenvironmental component required for SGC function and survival. In this study, we demonstrated a dose-dependent increase in viability of SGC after the application of an ECM overlay, which strongly supports the important role of ECM in regulating the survival or death of SGC. The remodeling of the complex structure of the SGC architecture after dissociation may rely on an ECM interaction to sense cellular environment. The ECM has been shown to facilitate survival in a number of cell types, and adhesion to ECM has previously been shown to conversely suppress apoptosis in several cell types (Boudreau, 2003; Fitzakerley, 2001). In the presence of ECM overlay ($\geq$200 μg/ml), a multilayered architectural structure was formed 72 h after plating. Hadley *et al.* (1985) reported their comparative study on co-culture systems cultured on tissue-culture–treated plastic, a matrix deposited by co-culture of Sertoli and peritubular myoid cells, and a reconstituted basement membrane gel from the EHS tumor. They found that co-cultures formed a polarized monolayer when grown on top of the reconstituted basement membrane, but that germ cells did not differentiate. They also indicated that when Sertoli cells were cultured within reconstituted basement membrane gels (3-D matrix) Sertoli and germ cells reorganized into cords, which can provide an environment permissive for germ cell differentiation. They suggested that such 3-D matrices effectively induced differentiated cell function better than traditional 2-D substrates. However, because of the high cost of culturing such cells within ECM gels, it is difficult to apply this culture system widely. Thus, our modified overlay culture system is a simpler and more cost-effective alternative method which facilitates cell–cell and cell–matrix interactions of the SGC, and which more mimics the 3-D structure as observed in *in vivo*.

Previous studies have demonstrated the significant role played by ECM in primary hepatocyte culture (Berthiaume *et al.*, 1996; Sidhu *et al.*, 1993, 2004). Application of the ECM overlay technique has resulted in a significant improvement in the expression levels as well as drug-inducibility of various cytochromes P-450 in primary hepatocytes (Sidhu *et al.*, 1993). Furthermore, a recent study (Sidhu *et al.*, 2004) has extended such observations with critical findings demonstrating that ECM modulation of cellular stress in primary hepatocyte cultures is intimately linked to improvements in both liver-specific and drug-inducible gene expression. The mechanisms by which ECM regulates cell growth, differentiation, and gene expression, especially for the Sertoli cells and gonocytes, are not yet fully defined. Cell–ECM interactions can directly regulate cell behavior, either through receptor-mediated signaling or by modulating the cellular response to growth factors (Klinowska *et al.*, 1999). The ECM interacts

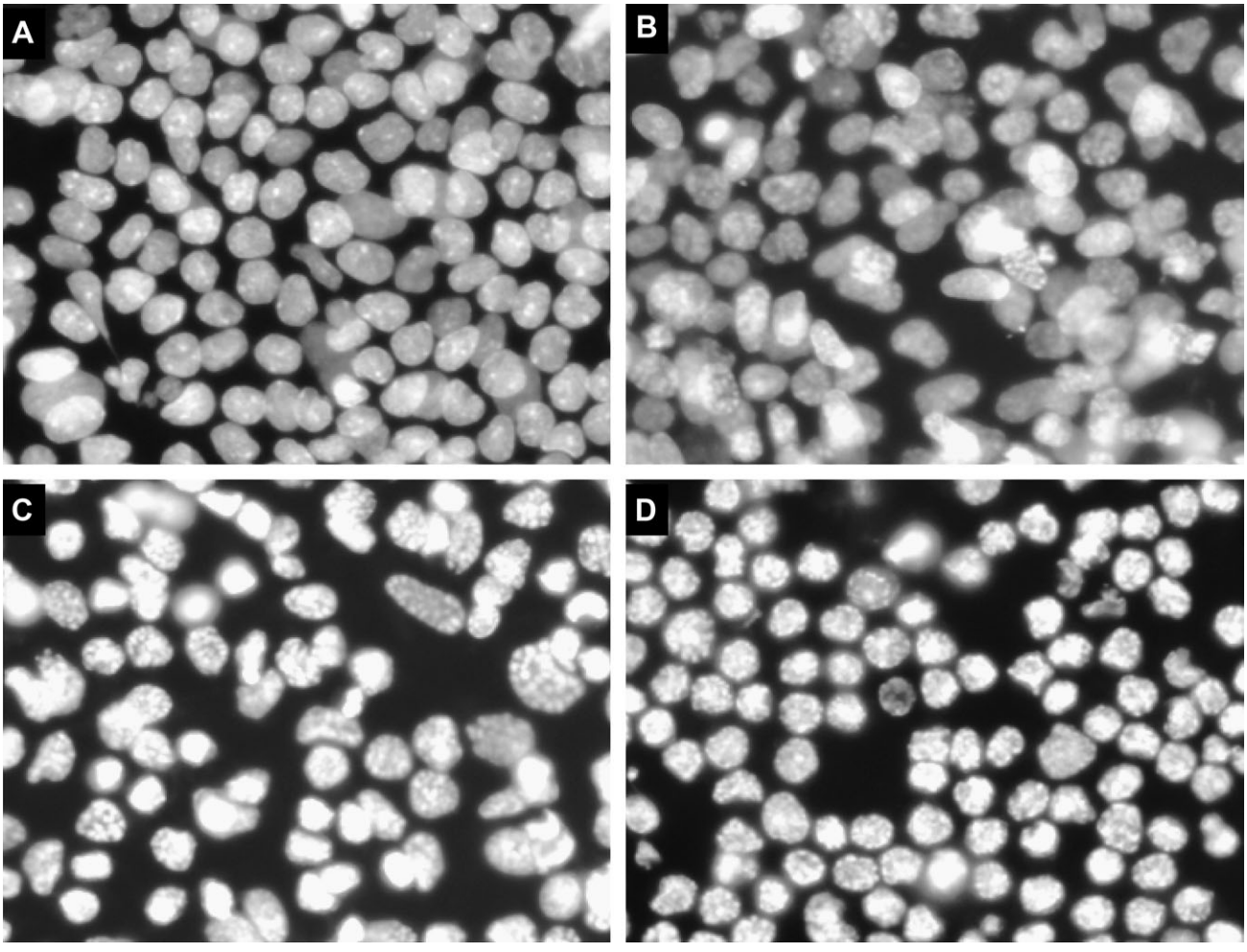


FIG. 10. Cd-induced dose-dependent apoptotic morphological alteration (A–D) and activation of caspase 3/7 activity (E). For the evaluation of the apoptotic morphological changes, cells were fixed and stained with Hoechst 33342 (0.1 mg/ml in PBS) 24 h after Cd treatment. The stained co-cultures were viewed with appropriate filters under fluorescent microscope. The image was captured and digitized with a Spot camera (Diagnostic Instrument, Inc) equipped with MetaMorph software. The activity of caspase 3/7 was measured by a fluorometric assay at 8 h and 24 h after treatment, with Ac-DEVD-AMC as the specific substrates. Fluorescent units were converted to *p* mol of AMC released per 10 μg of protein and incubation time (h) using a standard curve generated with known serial dilutions of AMC. Three replicates in three separate experiments were included.

directly with cell surface receptors such as integrins, the major class of ECM receptors that initiate signal-transduction pathways, which in turn modulate cell survival, growth, and apoptosis (Brassard *et al.*, 1999; Kanda *et al.*, 1999). The MAPK family pathways are well-defined by their respective downstream proline-directed protein kinases, including extracellular responses kinase (ERK1/2), stress-activated protein kinase/c-Jun amino-terminal kinase (SAPK/JNK), and p38 MAPK (Bhowmick *et al.*, 2001; Crean *et al.*, 2002; Rosen *et al.*, 2002). Mitogenic stimulation of the ERK1/2 pathway modulates the activity of many transcription factors, leading to biological responses such as proliferation and differentiation (Johnson and Lapadat, 2002). In contrast, the p38 MAPK and SAPK/JNK pathways were strongly activated by stress stimuli (Johnson and Lapadat, 2002; Rincon *et al.*, 2000) and are originally identified as an important pathway in the transduction of apoptotic signals initiated by stress or toxic stimuli such as ultraviolet light, osmotic stress, and inflammatory cytokines (Ichijo, 1999). In the present study, the ECM-mediated reduction of the stress signaling pathway, SAPK/JNK, and concomitant reduction of cell death, suggests that phosphorylation of SAPK/JNK signaling was involved in the activation of apoptosis, and that ECM plays an important role in modulating cellular stress signaling in neonatal Sertoli cell/gonocytes. Furthermore, we observed two different patterns of activation by the ECM of ERK1/2, an inverse dose-dependent activation at the early time point after plating and dose-dependent activation at later time

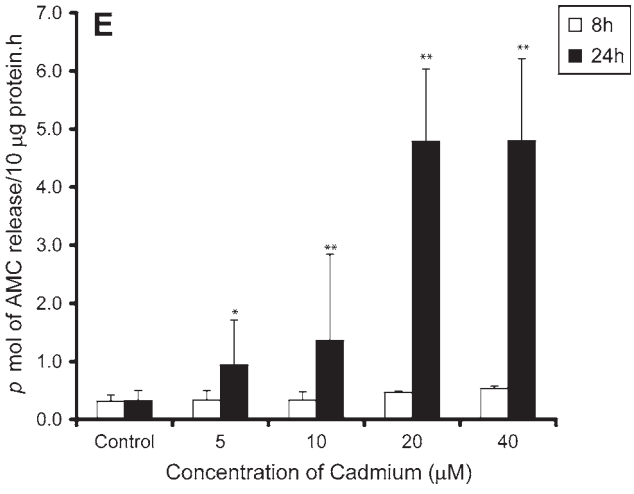


FIG. 10—*Continued.*

points. In the absence of or at low concentrations of ECM overlay and at the early time points post plating, cells may attempt to use rescue mechanisms such as the activation of ERK1/2 to maintain the integrity of the co-culture. However, in the absence of or at low concentrations of ECM overlay, the activation of the cellular rescue machinery is not sufficient to modulate the normal intracellular signal transduction response. As a result, increased cell death was observed as the activation of ERK1/2 decreased significantly 8 and 24 h later. Such findings suggest that, although activation of ERK1/2 was involved in cell survival, it by itself is not sufficient to maintain Sertoli cell/gonocyte function.

Protein kinase B (PKB/AKT) is activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components (Gu *et al.*, 2002). Protein kinase B phosphorylates and regulates the function of many cellular proteins involved in processes that include metabolism, apoptosis, and proliferation (Datta *et al.*, 1999; Ibuki and Goto, 2000). The intrinsic capacity of all cells to undergo apoptosis is suppressed by survival signals induced by factors within their immediate microenvironment. In this study, we observed that activation of AKT was critically linked with the dose of ECM as well as the duration that cells were exposed to the overlay. In the absence of an ECM overlay, the activation of AKT was significantly lower than in the presence of an ECM overlay. Previous studies have also shown that AKT-mediated survival is triggered by growth factors, extracellular matrix, and other stimuli (Datta *et al.*, 1999), and our current study suggests the indispensable role of ECM in regulating this response in the primary SGC co-culture.

Organization of the actin cytoskeleton during cell adhesion clearly requires intracellular signals that trigger polymerization of cytoskeleton proteins in response to all matrix adhesion (Berthiaume *et al.*, 1996; Machesky and Hall, 1997). In our study, the ECM overlay caused a dramatic reorganization of the cytoskeleton of SGC with the dose-dependent structural changes further confirming the significant role of the ECM overlay in reorganizing cellular architecture. An ECM overlay >200 μg/ml (Fig. 4D) appeared to be sufficient for the cells to adopt *in vivo*-like 3-dimensional diffusive cytoplasmic staining of FITC-phalloidin with a distinct line of intense staining where cells are in intimate contact with each other. Our current study strongly supports the notion that an ECM overlay is necessary in modulating the intracellular transduction signaling and reorganization of SGC structural integrity.

Finally, we evaluated the effects of ECM overlay on the expression level of c-Kit. The *c*-Kit proto-oncogene encodes for a plasma membrane-associated receptor with inherent tyrosine kinase activity. This receptor and its ligand, Stem Cell Factor (SCF) are vital for normal hematopoiesis, melanogenesis and gametogenesis (Orth *et al.*, 1997; Rossi *et al.*, 2000). Substantial evidence indicates that SCF is manufactured by Sertoli cell and that *c-kit* is expressed by germ cells during fetal development and in pubertal and adult rats. Interactions between this receptor on gonocytes and its ligand on the Sertoli cell surface are important in maintaining proliferation and differentiation of neonatal gonocytes (Rossi *et al.*, 2000). The ECM-mediated stabilization of c-kit protein expression indicated that the ECM overlay increased the interaction between Sertoli cells and spermotogonia at high concentrations, which supports the critical role of ECM in modulating the functional interaction between the Sertoli cells and gonocytes.

Cadmium is a ubiquitous environmental pollutant, and it has been reported to have male reproductive toxicity both in humans and in animals (IARC, 1976; Laskey *et al.*, 1986; Ragan and Mast, 1990; Schrag and Dixon, 1985). In the present study, we demonstrated a dose-dependent cytotoxicity and disruption of SGC structure after Cd treatment. The application of a three-color fluorescence assay in this *in vitro* culture system, combining with the recent advanced High Content Screening (HCS) Reader will provide a high throughput, automated solutions for testicular cell–specific screening for toxicity and activity of cellular constituents (Gaspari *et al.*, 2004). Our initial observation further revealed the induction of apoptosis at concentrations as low as 5 μM Cd, which suggests its potential in the mechanistic study of testicular toxicity.

In conclusion, our findings demonstrate that application of an overlay of ECM (≥200 μg/ml) to SGC permitted the formation of a testicular-like multilayered architectural structure that mimics *in vivo* characteristics of seminiferous tubules. The presence of an ECM overlay resulted in a dose- and time-dependent suppression of the stress-signaling pathway (SAPK/JNK) and conversely increased signaling responses (ERK1/2 and AKT) associated with the survival of spermatogonia. Likewise, the concomitant upregulation of the expression of c-Kit protein confirmed functional integrity of this co-culture system. These results support our proposal

that the ECM overlay enables a physiologically more stable SGC system. This system will therefore prove invaluable in the screen for testicular cell–specific cytotoxicity and in the examination of the molecular mechanisms associated with developmental and reproductive perturbations induced by environmental toxicants to Sertoli cells and/ or spermatogonial cell proliferation and differentiation.

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