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Differentiation of Human Trophoblast Cells In Vitro Is Inhibited by Dimethylsulfoxide

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Abstract Dimethyl sulfoxide (DMSO) exerts a number of biological effects, the most frequently cited being induction of cell differentiation. The compound also increases invasiveness and metastatic potential. In contrast to the many reports of DMSO-induced cell differentiation, we report here that DMSO inhibits the morphological differentiation of human cytotrophoblast cells to syncytiotrophoblast, as revealed by immunofluorescence staining for desmosomal protein and nuclei. Cytotrophoblast cells treated with DMSO under differentiation-inducing conditions remained mononucleated with intense desmosomal staining. The effect was dose dependent, with a maximal effect seen at 1.5% DMSO. Concentrations of ≤0.5% had no effect and concentrations >2% were cytotoxic. In addition to these morphological changes, DMSO inhibited secretion of human chorionic gonadotropin in a dose-dependent manner. At a concentration of 1.5%, DMSO inhibited secretion by 70%. If cytotrophoblast cells were cultured in the presence of DMSO and then switched to DMSO-free medium, they proceeded to differentiate normally. While the precise mechanism of action remains unknown, judicious use of DMSO may be a useful tool for studying and manipulating the differentiation of human trophoblast cells in vitro. The findings also indicate that care should be used in interpreting results obtained using DMSO as a carrier in drug and inhibitor studies. J. Cell Biochem. 65:460–468.

Key words: placenta; planar-polar compounds; hCG

INTRODUCTION

Dimethyl sulfoxide (DMSO) has many effects on biological systems and is also frequently used as a solvent or carrier for drugs and as a cryoprotectant for the storage of cells. One effect of DMSO that is seen with consistency in a variety of primary and continuous cell cultures is induction of differentiation [Omary et al., 1992; Depraetere and Joniau, 1995; Kuliczkowski et al., 1995; Teraoka et al., 1996]. While a common mechanism may be suspected based on the similarity of the cellular response, this has not been identified. In fact, a variety of biochemical responses to DMSO have been reported. DMSO causes changes in the expression of cell surface proteins (CD4, CD44, and KS-1) in colonic epithelial cells [Omary et al., 1992] and in the expression of the cell adhesion molecule, sialyl-Le^x, in a gastric adenocarcinoma cell line [Maehara et al., 1993]. Exposure to DMSO also increases the adhesion of adenocarcinoma cells to endothelium [Maehara et al., 1993]. Transient increases in intracellular Ca^{2+} concentrations are seen in several cell types upon exposure to DMSO [Morley and Whitfield, 1993]. DMSO-mediated induction of promyelocytic leukemia cell differentiation was accompanied by activation of a Na-dependent nucleoside transport system [Lee et al., 1994]. Wortmannin, an inhibitor of PI-3 kinase, blocks the DMSO-induced differentiation of murine erythroleukemia cells [Ai et al., 1995], suggesting a role for PI-3 kinase in this differentiation event. However, the precise molecular effect of DMSO in each of these cases is unknown. In spite of this, DMSO has proved a widely used and convenient agent for studying erythroid differentiation.

In humans, the placental syncytiotrophoblast interfaces directly with maternal blood and has many functions that are vital to success-

Abbreviations: 7-AAD, 7-aminoactinomycin D; DMSO, dimethylsulfoxide; hCG, human chorionic gonadotropin; HMBA, N,N′-hexamethylenebisacrylamide

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ful pregnancy outcome. Syncytiotrophoblast manufactures and secretes several hormones and cytokines and expresses a variety of different receptors that bind physiologically important ligands in maternal serum. However, the factors that regulate the differentiation of mononuclear cytotrophoblast cells to multinucleated syncytiotrophoblast are poorly understood. Epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and extracellular matrix (ECM) proteins appear to play a role in controlling trophoblast differentiation [Coutifaris et al., 1991; Kao et al., 1988; Morrish et al., 1987, 1991] and studies with colchicine indicate that microtubules are also important [Douglas and King, 1993]. In the present paper we show that, in contrast to its differentiation-inducing effects on other cells, DMSO inhibits the differentiation of cytotrophoblast to syncytiotrophoblast in vitro.

MATERIALS AND METHODS

Materials

DMSO (tissue culture grade) and antibody to desmosomal protein were obtained from Sigma Chemical Co. (St. Louis, MO). Antinuclear autoantibodies were obtained from Chemicon (Temecula, CA). TRITC- and FITC-labeled second antibodies were obtained from Accurate Chemicals (San Diego, CA). Keratinocyte growth medium (KGM) was obtained from Clonetics (San Diego, CA). 7-Aminoactinomycin D (7-AAD) and ethidium bromide were obtained from Molecular Probes (Eugene, OR), N,N′-Hexamethylenebisacrylamide (HMBA) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and stock solutions prepared in deionized water.

Trophoblast Isolation and Primary Culture

A detailed description of the procedure used to isolate cytotrophoblast cells from term human placentas has been given previously [Douglas and King, 1989]. Briefly, the method described by Kliman et al. [1986] was modified by the substitution of a continuous Percoll gradient (yielding >95% cytotrophoblast, as assessed by intermediate filament immunocytochemistry) and the addition of a final step, using immunomagnetic microspheres to remove the few remaining vimentin-positive and HLA-positive cells (yielding 100% cytotrophoblast). Gradient-purified cells were used for most experiments reported here, since the initial studies showed that identical results were obtained using both preparations. Cells were cultured in 8-chamber plastic LabTek slides and maintained in keratinocyte growth medium (KGM), containing 10% fetal calf serum (FCS). This medium supports trophoblast differentiation [Douglas and King, 1990]. After 72 h in KGM, more than 90% of the cells are multinucleated, as determined by desmosomal and nuclear staining [Douglas and King, 1993]. Cells were sometimes maintained in Ham's/Waymouth's medium (HWM), in which case they remained as mononucleated cytotrophoblast [Douglas and King, 1990]. For DMSO treatment, cells were suspended in medium containing the required concentration of DMSO (0.1–3.0% v/v) and then plated in LabTek Slides as described above. Unless stated otherwise, DMSO was present throughout the culture period.

Immunocytochemistry and Assessment of Trophoblast Differentiation

Cells were fixed and permeabilized using ice-cold methanol, after which they were incubated in PBS/gelatin. Cell–cell borders and nuclei were revealed by staining cultures simultaneously with an antibody against desmosomal proteins and an antinuclear autoantibody. Details of the indirect immunofluorescence staining procedure were described previously [Douglas and King, 1990]. In some experiments, nuclei were stained by incubating fixed and permeabilized cells for 30 min with 7-AAD (10 μg/ml) [Scholz et al., 1996]. Results were identical to those obtained using the antinuclear antibody (ANA). Stained cultures were examined using a Leitz Diaplan microscope with epifluorescence optics. Total numbers of nuclei and nuclei present as three or more within a single cell were counted with the aid of an eyepiece reticle. Three random fields were examined per well. At least 150 nuclei were counted per field.

Scanning Electron Microscopy

For evaluation by scanning electron microscopy (SEM), cytotrophoblast cells were plated onto glass coverslips and cultured in the presence or absence of DMSO (1.0%) for 3 days. The cells were rinsed once and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1.5 h. Dehydration was accomplished by taking the cells through a graded series of acetones, after which they were critical point dried out of

liquid CO_2. Cells were coated with a thin layer of gold–palladium before viewing in a Philips 501 scanning electron microscope.

Human Chorionic Gonadotropin ELISA

Human chorionic gonadotropin (hCG) released into cell culture medium was assayed using a two-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The method used was a modification of that described by Rapak and Szewczuk [1993]. Nunc plastic 96-well microtiter plates were coated by incubation for 2 hr with a purified monoclonal antibody (MAb) against hCG-β (1 µg/well; Fitzgerald Industries International, Concord, MA) and then blocked with casein, 2 mg/ml in phosphate-buffered saline (PBS), overnight. After washing the plate with PBS, samples of culture media to be analyzed were added (50 µl/well) and incubated for 2 h at room temperature (RT). After washing with PBS, a monoclonal anti-hCG antibody conjugated with horseradish peroxidase (HPO) (Fitzgerald Industries International) was added (100 µl; 0.1 µg/ml) and incubated for 2 h at RT. After washing, TMB substrate (3,3′,5,5′-tetramethylbenzidine; Fitzgerald Industries International) was added. The reaction was stopped by the addition of 0.1 M sulfuric acid, and the yellow color was read at 409 nm, using a Dynatech plate reader. The amount of hCG-β in samples has been expressed in IU and was determined by reference to a standard curve obtained using dilutions of a purified hCG (Fitzgerald Industries International).

Cell Viability

The viability of cells after incubation with DMSO for 72 h was assessed using ethidium bromide to stain dead cells. Briefly, cells were incubated with ethidium bromide (10 µM) for 10 min at RT and then washed, and the culture dish was read using a fluorescence plate reader. A parallel series of cell samples were assayed for protein content using a modified Lowry assay [Bennett, 1982]. Results are expressed as fluorescence units/µg protein.

Expression of Results

Results are expressed as means ± SD from at least three separate experiments. Statistical analyses were performed using Student's t-test. Micrographs are representative of results obtained from three separate experiments.

RESULTS

Effect of DMSO on Trophoblast Differentiation

When cytotrophoblast cells are plated in KGM, they differentiate, forming multinucleated syncytiotrophoblast-like masses within 48–72 hr. We have described this in detail previously [Douglas and King, 1990]. The syncytiotrophoblast-like masses are readily discerned by staining the cultures with antibodies against nuclei and desmosomal proteins, so revealing nuclei and cell borders. Figure 1A shows a typical colony grown in KGM with many nuclei within a common cytoplasm, typical of syncytiotrophoblast. Figure 1B shows a trophoblast culture that had been maintained in HWM for 3 days and stained in the same way. In contrast to the cells maintained in KGM, there is strong staining of cell–cell borders by the antidesmosomal antibody, and it can be seen that most of the cells are mononucleated, typical of cytotrophoblast. This has also been described previously [Douglas and King, 1990].

Figure 1C–F shows trophoblast cells cultured in KGM in the presence of DMSO (0.25–1.5% v/v). At final concentrations of 0.25 or 0.5% (Fig. 1C,D), the staining pattern was the same as cells maintained in KGM in the absence of DMSO (Fig. 1A). In other words, cultures consisted of large multinucleated colonies. In contrast, at DMSO concentrations of 1.0% and 1.5% (Fig. 1E,F), the cultures showed strong desmosomal staining at cell–cell borders and most of the cells were mono- or binucleate, similar to cells maintained in HWM (Fig. 1B).

We next attempted to quantitate the effects of DMSO on trophoblast differentiation. After culturing cells in KGM in the presence of different concentrations of DMSO for 72 h, followed by immunocytochemical staining as described above, nuclei were counted and the percentage of nuclei present in multinucleated cells calculated. The results in Figure 2 show that control cultures incubated in KGM in the absence of DMSO consisted of 93% multinucleated cells. The number of multinucleated cells in cultures incubated in the presence of 0.25 or 0.5% DMSO was not significantly different from the KGM control. However, cultures incubated in the presence of 1.0% or 1.5% DMSO consisted of only 26% or 17% multinucleated cells, respectively. These latter results are similar to the value obtained for trophoblast maintained in HWM (i.e., nondifferentiated cytotrophoblast).

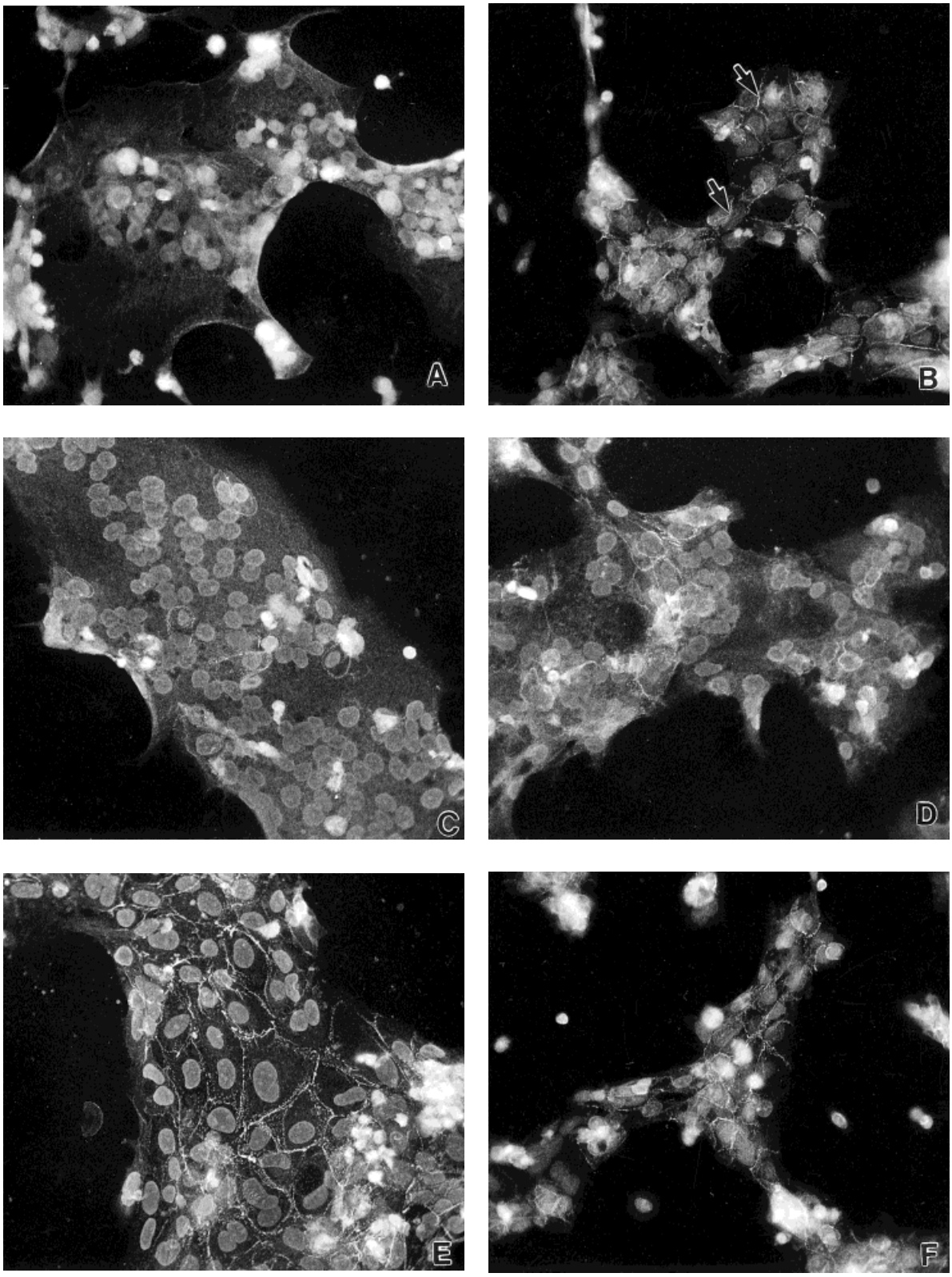

Fig. 1. Effect of DMSO on syncytiotrophoblast formation. For these studies, trophoblast cells were cultured for 3 days in the presence or absence of different concentrations of DMSO. They were then stained using antidesmosomal protein and antinuclear antibodies as described in the methods section. **A:** Cells cultured in KGM in the absence of DMSO. **B:** Cells maintained in HWM in the absence of DMSO. **C–F:** Trophoblast cells cultured in KGM in the presence of DMSO (0.25, 0.5, 1.0, and 1.5%, respectively). Epifluorescence, ×310. *Arrows,* desmosomal staining.

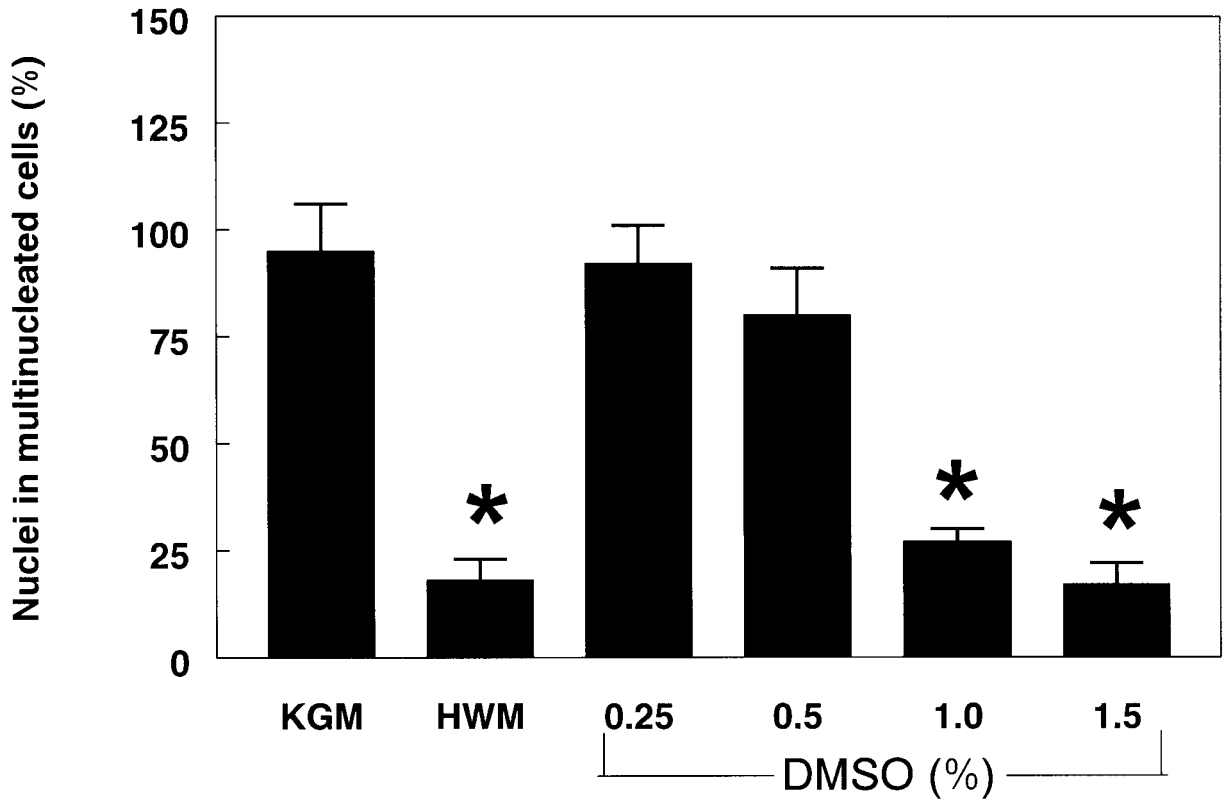

Fig. 2. Quantitation of syncytiotrophoblast formation in the presence of DMSO. Trophoblast cells were maintained in KGM in the presence of 0.25%, 0.5%, 1%, or 1.5% DMSO for 72 h. After staining to reveal nuclei and cell–cell borders, nuclei were counted and the percentage of nuclei present in multinucleated cells was calculated (see Methods). Results are expressed as means ± SD from three separate experiments. *Asterisks,* values that were significantly different ($P < 0.05$) from the KGM control.

In order to determine whether trophoblast differentiation could be blocked by other polar compounds, cells were incubated in the presence of different concentrations of HMBA. At concentrations of 10 mM and 2 mM, HMBA blocked the formation of syncytiotrophoblast as revealed by desmosomal/nuclear staining. At 0.5 mM, HMBA was not effective and the cells differentiated normally (results not shown).

Effect of DMSO on Trophoblast Viability

In this assay, dead (but adherent) cells were detected by ethidium bromide staining of nuclei and quantitated using a fluorescence plate reader (Fig. 3). Results are expressed as arbitrary fluorescence units/μg cell protein. Control trophoblast cultures gave readings of about 20 fluorescence units/μg, which is equivalent to a viability of 87% (determined by counting nuclei). There was no significant loss of viability at DMSO concentrations of ⩽1.5%, but the number of dead cells increased at higher concentrations. Figure 3 also shows the cellular protein content of cultures treated with DMSO and so provides a measure of cell attachment (or cell detachment). DMSO at concentrations of ⩽1.5% had no effect on cell attachment but there was a significant loss of cells at higher concentrations.

We next sought to determine whether the effects of DMSO were reversible. Cells were plated in KGM in the presence of 1% DMSO and incubated for 16 hr. At this time the medium was removed and replaced with fresh medium containing no DMSO. The cells were then incubated for a further 72 h, then fixed and stained to reveal nuclei and cell–cell borders (Fig. 4A). It can be seen that these cultures consisted of large multinucleated colonies indicating that the cells differentiated after removal of the DMSO. This was substantiated by calculations that revealed that 87 ± 9% of nuclei were in multinucleated cells. This figure is similar to that for control differentiated cul-

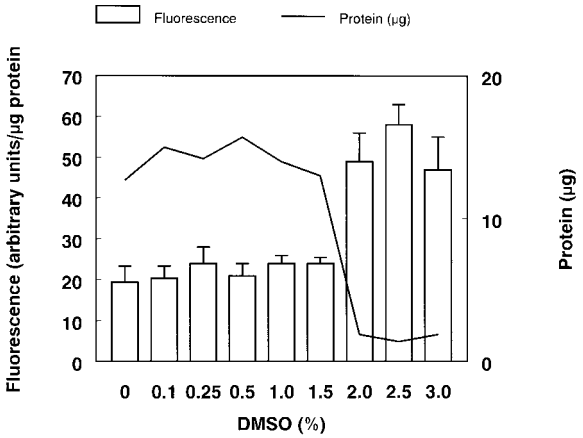

Fig. 3. Effect of DMSO on trophoblast viability. Cells were incubated in the presence or absence or different concentrations of DMSO for 72 h. The cultures were then incubated with ethidium bromide for 10 min, washed, and the plates read in a fluorescence plate reader. A parallel series of cell cultures were used for protein determination. The bar chart shows arbitrary fluorescence units/μg protein. The superimposed line graph shows the cellular protein content. Control trophoblast cultures gave readings of about 20 fluorescence units/μg, which is equivalent to a viability of 87%, based on examination of stained cultures by fluorescence microscopy. Results are means ± SD from three experiments.

tures incubated in the absence of DMSO (cf. Fig. 2). Figure 4B shows a control culture incubated in the presence of DMSO for the entire culture period where, as expected, the culture consists mostly of cytotrophoblast-like cells (26 ± 6% of nuclei were in multinucleated cells).

As an additional means of assessing the possible effects of DMSO on trophoblast viability, we compared DMSO (1.0%)-treated trophoblast and control cytotrophoblast cultures by SEM. The cultures consisted of flattened, adherent cells and were indistinguishable (Fig. 5). Specifically, the apical plasma membranes of both cultures showed an abundance of microvilli, further attesting to the integrity of the cells in the presence of DMSO.

Effect of DMSO on hCG Secretion

In this experiment cells were incubated in the presence or absence of DMSO for 72 h after which the culture medium was assayed for hCG-β. The results displayed in Figure 6 show that DMSO inhibited the secretion of hCG in a dose-dependent manner. Significant effects were seen at DMSO concentrations of ⩾0.5%. At the highest concentration tested (1.5%), DMSO reduced hCG secretion by about 70%, as compared to differentiated control (KGM) cultures.

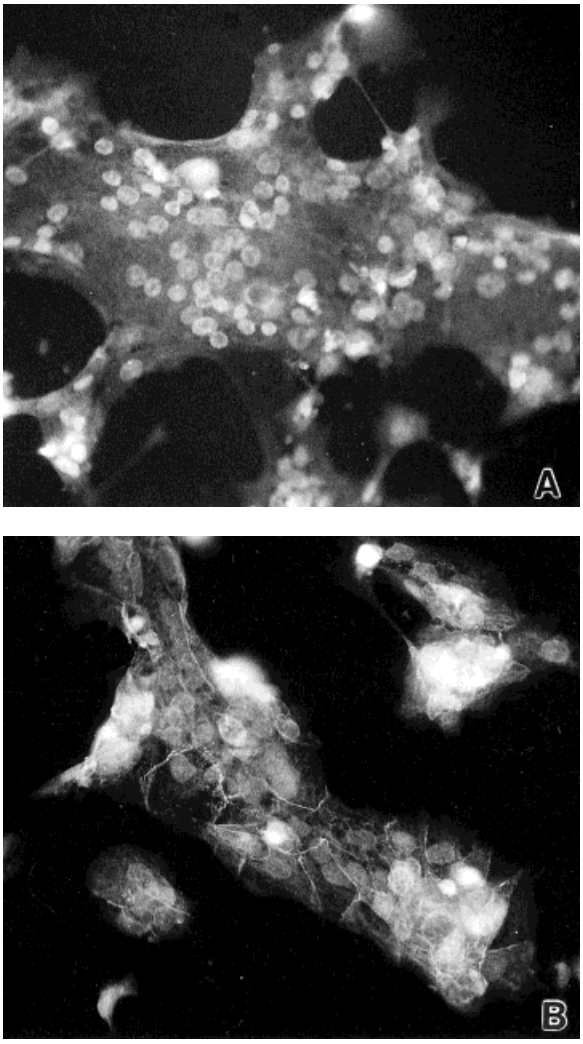

Fig. 4. The effects of DMSO are reversible. Cells were plated in KGM in the presence or absence of 1.0% DMSO and incubated for 16 h. The medium from the DMSO-treated cultures was then removed and replaced with fresh medium containing no DMSO. The cells were then incubated for a further 72 h, after which they were fixed and stained to reveal desmosomes and nuclei **(A). B:** A culture incubated in the presence of DMSO for the entire culture period. Epifluorescence, ×310.

This reduction in secretion was not an artifact caused by increased secretion of proteases, since incubation of spent culture medium from either DMSO-treated or control trophoblast cultures or mixtures of both at 37°C for 24 h showed no loss of hCG immunoreactivity (result not shown). If cultures were incubated in KGM in the presence of 1.0% DMSO for 16 h and then reincubated in fresh KGM in the absence of DMSO for 72 h hCG secretion increased to control levels (Fig. 6, WASH).

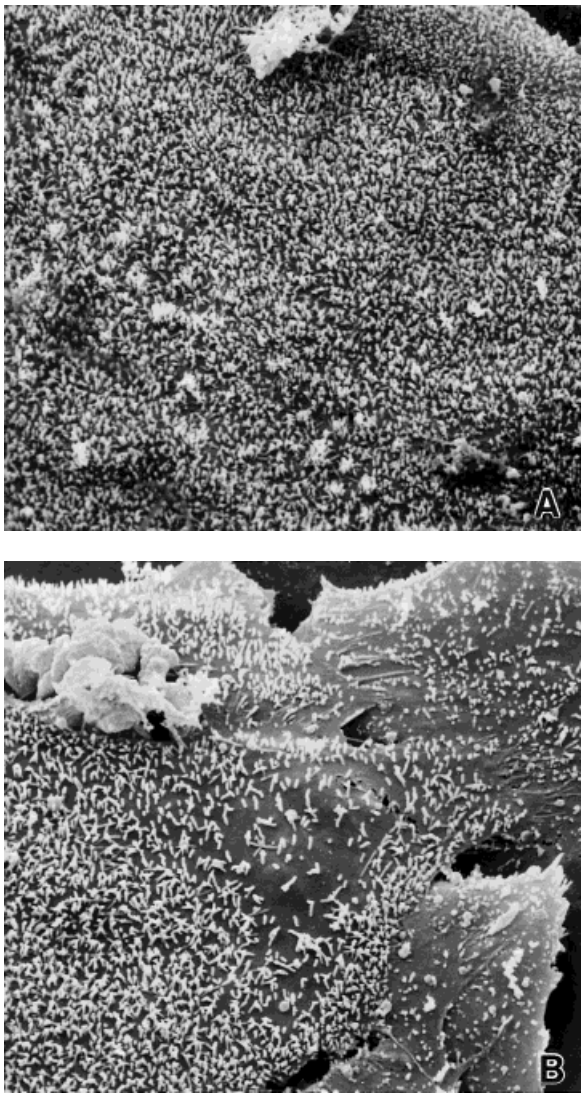

Fig. 5. Scanning electron microscopy of DMSO-treated cultures. **A:** Micrograph of trophoblast culture after 72-h exposure to 1.0% DMSO. **B:** Micrograph of control cytotrophoblast culture incubated in HWM in the absence of DMSO. ×2,500.

DISCUSSION

Although it is now generally agreed that the formation of syncytiotrophoblast arises by the fusion of mononuclear cytotrophoblast cells, the factors that control this process are poorly understood. The polar–planar solvent DMSO is frequently used to induce erythroid differentiation in vitro [Chakravarthy et al., 1992; Collins et al., 1978; Ai et al., 1995]. It also causes changes in the growth and differentiation of other transformed cell lines [Kim et al., 1980; Omary et al., 1992]. Because of its well-known differentiation-inducing effects and because it is frequently used as a solvent or carrier for drug studies, we decided to investigate the effects of DMSO on human trophoblast differentiation.

In contrast to findings in other cells, we found that DMSO inhibits the differentiation of cytotrophoblast cells to syncytiotrophoblast. This conclusion is based on the concentration-dependent inhibition of multinucleated cell (syncytiotrophoblast) formation and on the concentration-dependent inhibition of hCG secretion. The concentrations of DMSO that were found to be effective in the present study (1.0–1.5%) are within the concentration range reported to be effective in other cell systems. At these concentrations, there was no loss of cell viability. Treated cells had normal morphology and well-developed apical brush borders, as assessed by fluorescence microscopy and scanning electron microscopy. These observations further attest to the noncytotoxic effects of DMSO at concentrations of $\leqslant$1.5%. It should be noted, however, that at higher concentrations ($>$1.5%) DMSO caused a significant loss of trophoblast viability. In other systems, concentrations of $\leqslant$3% are frequently tolerated without toxic effects. The effects of noncytotoxic doses of DMSO on trophoblast cells required that the drug be present throughout the culture period; removal of DMSO allowed the cells to differentiate as revealed by the formation of multinucleated cells and by increased secretion of hCG.

The mechanism by which DMSO inhibits trophoblast differentiation is not known. Similarly, the mechanism of action of DMSO in those cells where it acts as a differentiation inducer is also unclear. Despite studies showing that DMSO affects a variety of cellular functions [Yen and Varvayanis, 1995; Ai et al., 1995; Chakravarthy et al., 1992; Morley and Whitfield, 1993], no specific target molecule has been identified. However, the polar nature of DMSO and the fact that its biological effects are seen at relatively high concentrations provide some fuel for speculation. Furthermore, another polar compound, N,N′-hexamethylenebisacrylamide, also blocked trophoblast differentiation. Thus, while it is possible that the observed effects result from the direct interaction of DMSO with a specific cellular component, it is more likely that DMSO (and other planar–polar compounds) interacts with low specificity with several cellular components. It is unclear why DMSO inhibits the differentiation of trophoblast cells while inducing differen-

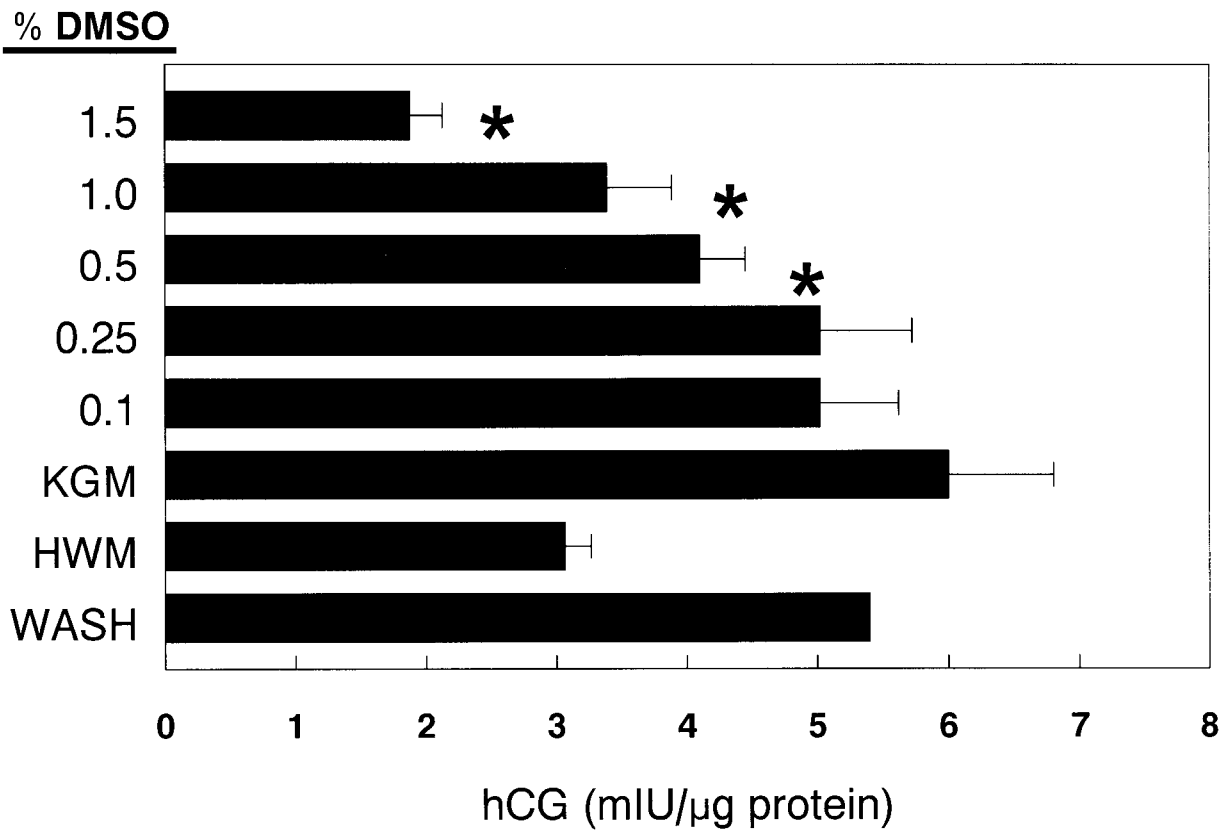

Fig. 6. Effect of DMSO on hCG secretion. Cells were incubated in the presence or absence of different concentrations of DMSO for 72 h, after which the culture medium was collected and assayed for hCG-β using a two-antibody ELISA. Results are means ± SD from four experiments. The asterisks indicate values that were significantly different ($P < 0.05$) from the KGM control. In another experiment (WASH), cells were incubated in KGM in the presence of 1.0% DMSO for 16 h and then reincubated for 72 h in KGM without DMSO. hCG was then assayed as above. In this case, the value for hCG secretion is the mean of two separate experiments.

tiation in other cell systems. Induction of erythroid cell differentiation by DMSO begins with cessation of cell replication followed by a commitment phase and then the structural and functional changes that result in terminal differentiation. Cytotrophoblast cells from term placentas show no replication when cultured in vitro and terminal differentiation is unusual in that it involves cell–cell fusion to form large multinucleated syncytiotrophoblast cells. It is generally agreed that DMSO destabilizes membranes at physiological temperatures while having a stabilizing effect at freezing temperatures [Anchordoguy et al., 1992; Arakawa et al., 1990]. With this in mind one would perhaps expect DMSO to have induced rather than inhibited the cell–cell fusion that accompanies trophoblast differentiation. Clearly, further experiments are required before the effect of DMSO on this process can be explained.

In spite of the lack of information about the mechanism of action of DMSO on trophoblast differentiation, the findings presented here have other important implications. First, the fact that DMSO is frequently used as a carrier for drug studies indicates that data should be analyzed carefully and possible effects due to the carrier ruled out by the inclusion of appropriate controls. Potential problems can also be avoided by ensuring that final DMSO concentrations are <0.5%. Second, the consistent, noncytotoxic and reversible effects of DMSO provide a new and useful tool for studying and manipulating trophoblast differentiation in vitro.

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