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Coordinated regulation of apoptosis and cell proliferation by transforming growth factor β1 in cultured uterine epithelial cells

(programmed cell death/uterus)

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ABSTRACT **Cell and tissue growth is regulated through a complex interplay of stimulatory and inhibitory signals. We describe two biological actions of transforming growth factor β1 (TGF-β1) in primary cultures of rabbit uterine epithelial cells: (*i*) inhibition of cell proliferation and (*ii*) a concomitant increase in cells undergoing apoptosis (programmed cell death). It is proposed that proliferation and apoptosis together comprise normal cell growth regulation.**

Transforming growth factor β1 (TGF-β1) has been shown to be a multifunctional growth-regulatory molecule that either stimulates mesenchymal or inhibits epithelial cell growth (1–6). In some experimental systems, that inhibition of growth is fully reversible (7, 8). In addition, Müllerian inhibiting substance, a glycoprotein closely related to the TGF-β family of molecules, is involved in apoptotic regression of the Müllerian ducts in reproductive organ development (9), and levels of TGF-β mRNA have been found to increase in the castration-induced apoptosis (also known as programmed cell death) of rat ventral prostate (10).

Previous reports have described both morphologically and biochemically the occurrence of apoptosis in rabbit uterine epithelium (11) and have catalogued the effects of 17β-estradiol and progesterone on cell death by morphological criteria in animals (12). Primary rabbit uterine epithelial cells grown on floating collagen gels in chemically defined medium exhibited morphological features of apoptosis, as well as regulation by soluble factors from conditioned medium, modulating either cell proliferation or cell death (13). A coordinated regulation of cell proliferation and apoptosis was proposed (11–13). To further investigate that hypothesis, we studied the effects of TGF-β1 on cell proliferation and apoptosis of primary uterine epithelial cell cultures.

MATERIALS AND METHODS

Cell Culture. Uterine epithelial cells isolated from uteri of mature rabbits were plated at 250,000 cells per 35-mm dish in chemically defined medium (14) supplemented with 0.05% heat-inactivated calf serum (HyClone). The day after plating, cells were washed twice with warm phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Medium was changed every 24 hr. DNA was quantitated by the Hoescht dye-binding technique and expressed as micrograms per dish (13). Recombinant TGF-β1 was purified from serum-free medium conditioned by CHO clone 17 cells and was stored in 4 mM HCl/0.1% BSA at 4°C (15). This growth factor was supplied by Oncogen (Seattle, WA) at 6.8 μM in 30% acetonitrile containing 0.05% trifluoroacetic acid. From this a 0.1 μM stock solution was made in 4 mM HCl/0.1% BSA (Sigma). Control cell cultures contained only the vehicle.

Apoptotic DNA Isolation and Quantitation. Uterine epithelial cells were plated at 750,000 cells per 60-mm dish in chemically defined medium supplemented with 0.05% heat-inactivated calf serum. The day after plating, cells were washed with PBS/0.1% BSA and incubated with recombinant TGF-β1 for 5 days. On day 5, cells were lysed *in situ* with 0.8 ml of digestion buffer (100 mM NaCl/10 mM Tris·HCl, pH 8.0/25 mM EDTA, pH 8.0/0.5% sodium lauryl sulfate/0.03% proteinase K) and incubated at 37°C overnight. DNA was extracted once with phenol and once with phenol/chloroform, 1:1. Samples were precipitated with 7.5 M ammonium acetate and 100% ethanol at −70°C. Precipitated material was rinsed with 85% ethanol and treated with DNase-free RNase (1 μg/ml) and 0.1% sodium lauryl sulfate for 1 hr at 37°C. Another phenol/chloroform, 1:1, extraction and a chloroform extraction were performed. DNA was precipitated a second time, dissolved in 10 mM Tris·HCl, pH 8.0/1 mM EDTA, and then analyzed with a DU-50 spectrophotometer at 260 nm and 280 nm. Twenty micrograms of DNA was loaded in each well and electrophoresed in a 1.5% agarose gel with 40 mM Tris acetate, pH 8.5/2 mM EDTA buffer. Agarose gels were stained with ethidium bromide (0.5 μg/ml) for 1 hr and photographed with an MP4 Polaroid camera employing UV transillumination and type 55 Polaroid film. Each negative was scanned with a Bio-Rad video densitometer, and the percentage of DNA fragments was expressed as area of fragmented DNA divided by total DNA area (fragmented plus high molecular weight DNA).

All statistics (one-factor analysis of variance) were performed by the STATS PLUS program (distributed by Jandel Scientific, Sausalito, CA) on an Apple IIe personal computer.

RESULTS AND DISCUSSION

We first observed the presence of apoptotic cells in primary cultures of uterine epithelial cells grown on plastic tissue culture dishes in chemically defined medium (Fig. 1) and discovered that addition of TGF-β1 induced significant decreases in total DNA per dish. The optimal growth-inhibitory effects of TGF-β1 were found to occur in cells cultured in the presence of 0.05% calf serum. Heating serum at 70°C or 100°C for 20 min or stripping with dextran-coated charcoal did not eliminate the TGF-β1 inhibitory effects. Different sera—human, rabbit, fetal and adult bovine—had similar effects when added at 0.05% (data not shown). The mechanism of action of serum in this case is unknown but may involve either growth factors or nutrients present in the serum (16). Fig. 2 shows that a 50% growth-inhibitory effect was ob-

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Abbreviations: TGF, transforming growth factor; BSA, bovine serum albumin.

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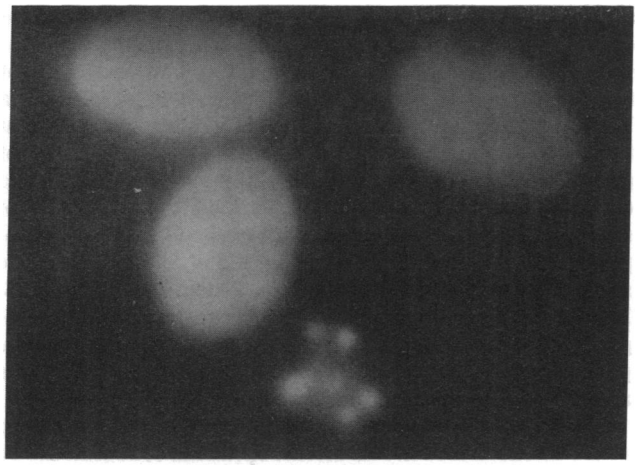

FIG. 1. Fluorescence micrograph shows one fragmented apoptotic cell nucleus and three intact nuclei. Primary cultured cells were grown for 5 days in control medium on tissue culture chamber/slides (Lab-Tek), fixed with cold 30% methanol/70% acetone, and stained for 30 min for DNA with Hoescht 33258 fluorochrome (0.1 μg/ml in distilled water; Behring Diagnostics). Slides were then mounted with Aquamount and coverslips. Photographs were obtained with an epifluorescence microscope. (×410.)

served with TGF-β1 at ≈80 pM, as determined by DNA measurements. Cultured cells that were incubated with TGF-β1 reinitiated cell growth within 6 days after withdrawal of TGF-β1 (Fig. 3), demonstrating reversibility of the TGF-β1-induced growth inhibition. Fig. 3 shows that long incubation with TGF-β1 results in decreased effects, suggesting a possible desensitization to this factor's inhibitory action.

Cell proliferation and cell death were studied simultaneously by determining the total DNA content per dish, death indices (percent apoptotic cells), and labeling indices (per-

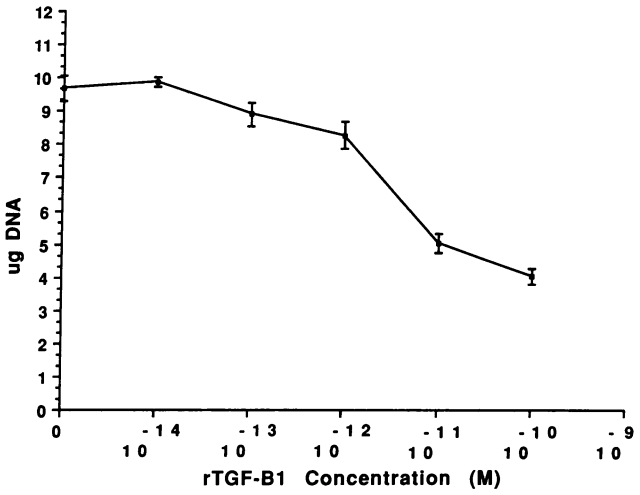

FIG. 2. Concentration-dependent growth-inhibitory effects of TGF-β1. The day after plating, selected concentrations of recombinant (r) TGF-β1 (10^{-14} to 10^{-10} M) were added to respective cultures. Vehicle only was added in control cultures. After 6 days, DNA was quantitated as μg per dish. Data represent means ± SEM (n = 5). Statistical comparisons are summarized below.

Comparison	F	P
0 vs. 10^{-14} M	4.64	0.0471
0 vs. 10^{-13} M	2.18	0.1820
0 vs. 10^{-12} M	6.46	0.0334
0 vs. 10^{-11} M	94.79	<0.001
0 vs. 10^{-10} M	145.49	<0.001

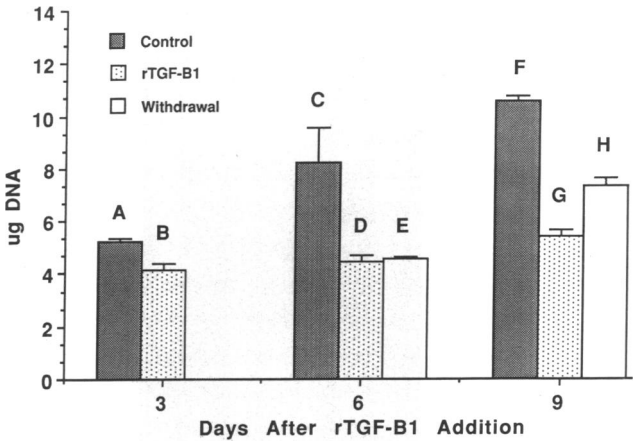

FIG. 3. Reversible growth-inhibitory effects of TGF-β1. Cultures were prepared as described, and incubated with recombinant (r) TGF-β1 at 0.1 nM after 2 days of culture in medium plus 0.05% calf serum. Media with rTGF-β1 were withdrawn in a group after 3 days, and medium containing only 0.05% calf serum plus vehicle buffer was added back to these cells. Data represent μg of DNA per dish (mean ± SEM, n = 5). Statistical comparisons are summarized below.

Comparison	F	P
A vs. B	15.08	0.0040
C vs. D	7.51	0.0246
D vs. E	0.22	—
F vs. G	329.57	<0.001
G vs. H	30.32	0.001
D vs. G	8.27	0.02

cent cells with labeled nuclei after incubation with [^{3}H]thymidine). Decreases in DNA and labeling indices, with concomitant increases in death indices, were evident in TGF-β1-treated but not in control cultures (Table 1). The determination of cell death in cultured cells may not reflect absolute values, since a number of apoptotic cells become nonadherent (unpublished data). Therefore, the observed death indices show only the apoptotic cells still attached.

In the process of apoptosis, nuclear DNA is cleaved into a DNA "ladder," reflecting cleavage of internucleosomal sites (17–19), which is generally associated with the apoptotic process. DNA from primary uterine epithelial cells cultured on plastic tissue culture dishes, when electrophoretically separated in an agarose gel, exhibits this phenomenon, as would be anticipated from the morphological observations. Fig. 4 illustrates that DNA isolated from cells cultured in the presence of TGF-β1 (lane +) was fragmented significantly more than DNA from control cells (lane −). Densitometric measurements of ethidium bromide-stained DNA bands con-

Table 1. Effects of TGF-β1 on cell proliferation and apoptosis

Addition(s) to medium	DNA, μg per dish	Labeling index	Death index
Serum	4.5 ± 0.2	37.2 ± 1.3	32.5 ± 1.6
Serum + TGF-β1	3.4 ± 0.1*	15.6 ± 1.7*	38.6 ± 2.3†

On day 6, DNA content (μg per dish, n = 5) and percentages of cells with labeled nuclei (labeling indices) as well as percentages of apoptotic cells (death indices) were determined in cell cultures incubated with 0.05% calf serum or 0.05% calf serum plus 0.1 nM TGF-β1. Cells were fixed with methanol 24 hr after addition of [^{3}H]thymidine (0.5 μCi/ml; 1 μCi = 37 kBq) and processed for autoradiography (13). Apoptotic cells were identified after staining with Gill's reagent. Five hundred cells per dish were blindly counted for labeling (n = 5) and death indices (n = 3). Data are expressed as mean ± SEM.

*$P < 0.001$ (F = 22.2).

†P = 0.01 (F = 9.8).

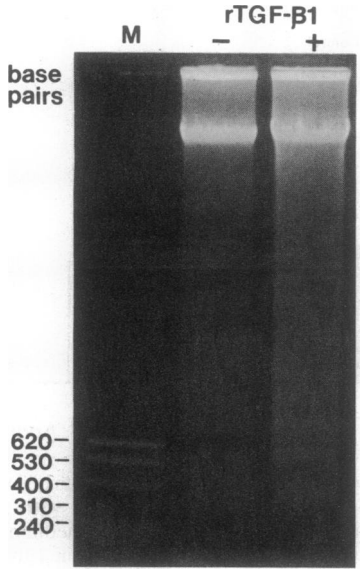

FIG. 4. Cells cultured with recombinant (r) TGF-β1 at 0.1 nM have increased levels of DNA fragmentation. On day 5 of culture, DNA was isolated and purified from uterine cells and analyzed for DNA fragmentation by agarose gel electrophoresis. Hoescht dye quantitation of DNA (μg per dish, mean ± SEM, $n = 5$): no rTGF-β1, 7.3 ± 0.4; plus rTGF-β1, 4.7 ± 0.1; $P < 0.001$ by one-factor variance analysis. Densitometric integration units represent DNA fragments scanned in each lane; no rTGF-β1, 18.7 (lane −); plus rTGF-β1, 29.6 (lane +). Lane M shows molecular size markers (*Hpa* II digest of pBR322 plasmid DNA).

firmed the visual observations. Our results show that TGF-β1 causes not only a decrease in cell proliferation but also an increase in apoptosis.

Apoptosis is a physiologic type of cell death recognizable at the light microscopic level by characteristic condensed basophilic cytoplasm, darkly stained karyorrhectic nuclei, and the presence of apoptotic bodies, which can be sloughed off or can be phagocytosed by macrophages or by neighboring cells. Biochemical analysis demonstrates that isolated nuclear DNA from primary uterine epithelial cells undergoing apoptosis is cleaved into a ladder of DNA fragments in multiples of approximately 200 base pairs. This chromatin cleavage is believed to be accomplished by a Ca^{2+}- and/or Mg^{2+}-dependent endonuclease (20–22). Apoptosis has been described extensively in embryonic development (23) and thymic maturation (24), where it plays important roles in deletion of specific cell populations. Its role in adult tissues has not been clarified. We are now proposing that apoptosis as well as proliferation are components of growth regulation in normal epithelia. Studies with animals (11, 12) and primary cultures (13) have shown that apoptosis in uterine epithelial cells is regulated in a coordinated but inverse manner with cell proliferation.

The reversibility of TGF-β1 effects described here is consistent with an earlier report (13) where it was proposed that stem-cell proliferation is regulated by a "cell proliferation factor(s)," while daughter cells are the target for a "cell death factor(s)" that induces apoptosis. It is possible that TGF-β1 may modulate or potentiate the level of response to the latter factor, which could also be a member of the TGF-β family. Alternatively, a model could be conceived in which there is only one target cell, having at least two sets of growth regulatory genes. One of them would code for signals regulating cell proliferation and the other for apoptosis. The same effector—i.e., progesterone—could differentially modulate both, or it could modulate only one set of genes, which would then in turn affect the other set. The ecdysteroids, a class of steroids controlling insect metamorphosis, have been described as the mediators of programmed neuron and muscle death in the moth *Manduca sexta* (25, 26). A causal relationship has been described between mutations in one region of the decapentaplegic gene of *Drosophila*, which codes for a TGF-β homolog, and localized apoptosis in structures lost from adult appendages (27). Studies on the genetics of programmed cell death in the development of the nematode *Caenorhabditis elegans* have described the existence of several genes that regulate autonomous cell death and apoptosis that occurs only in the presence of another cell. The same authors showed evidence for a stem-cell system (28, 29). The aforementioned data demonstrate crucial effects of growth-regulatory genes upon very complex developmental phenomena involving other genes, as well as evidence in agreement with our hypotheses.

Coordinated but inverse regulation of cell proliferation and death was described first in uterine epithelial cells cultured on collagen gels (13) and later in pseudopregnant rabbit uterine epithelium (11, 12). The present results provide further evidence for involvement of apoptosis in this type of growth regulation. It is interesting that growth studies using cell lines have addressed the phenomena of cell proliferation (2) and cell death (18, 30, 31) separately, but not both at the same time. We suggest that some established cell lines may have lost this type of coordinated regulation because they were selected on the basis of continuous proliferation. Thus, normal primary or early-passage cell cultures may be advantageous for addressing mechanisms of apoptosis.

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