Transforming Growth Factor-β1 Inhibits Nucleosomal Fragmentation in Human Keratinocytes following Loss of Adhesion*

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Kris F. Sachsenmeier, Nader Sheibani, Sandra J. Schlosser, and B. Lynn Allen-Hoffmann‡

From the Department of Pathology and the Environmental Toxicology Center, Madison, Wisconsin 53706

We have investigated the role of transforming growth factor-β1 (TGF-β1) in suspension-induced programmed cell death of cultured human keratinocytes. Suspension of keratinocytes in semisolid medium induces TGF-β1 mRNA levels and synthesis of bioactive TGF-β1 protein. Concomitant with the suspension-induced increase in secreted TGF-β1 levels, steady state mRNA levels for c-myc are decreased. Both exogenously added and endogenously produced TGF-β1 attenuate suspension-induced nucleosomal fragmentation in keratinocytes. We propose that TGF-β1 may function to protect keratinocytes from DNA fragmentation following loss of cell-substratum and/or cell-cell contact. Taken together, our findings suggest that loss of cell-substratum and/or cell-cell adhesion is an important component of an apoptotic signal transduction cascade regulated by TGF-β1 in normal human stratified squamous epithelia.

We have described the morphological and biochemical features of apoptosis in cultured normal human keratinocytes and identified loss of cell-cell and/or cell-substratum contact as an important signal for keratinocyte cell death in vitro. The pleiotropic molecule transforming growth factor β1 (TGF-β1) has been shown to be a potent modulator of the apoptotic process in a variety of cell types. In certain simple epithelial cell types and hematopoietic cells, TGF-β1 has been reported to induce programmed cell death (Oberhammer et al., 1992; Rotello et al., 1991; Lotem and Sacha, 1992). TGF-β1 also inhibits the growth of normal human keratinocytes in vitro (Pietenpol et al., 1990). Based on studies using normal human keratinocytes and the murine keratinocyte cell line, BALB/3K, it is known that TGF-β1 inhibits keratinocytes in the G1 phase of the cell cycle (Shipley et al., 1986) through a mechanism that most likely involves transcriptional down-regulation of c-myc expression (Pietenpol et al., 1990; Munger et al., 1992). In the present study, we identify TGF-β1 as an important inhibitor of apoptosis in cultured normal human keratinocytes. We propose that TGF-β1 may function as a signal to forestall apoptosis as well as replication in the basal layer of human skin.

**MATERIALS AND METHODS**

**Cell Culture**—Normal keratinocytes were isolated from newborn human foreskin. Keratinocyte cultures were established by plating aliquots of the single cell suspension in the presence of mitomycin C-treated 3T3 feeder cells as described by Allen-Hoffmann and Rheinwald (1984). The standard keratinocyte culture medium was composed of a mixture of Ham's F12/Dulbecco's modified Eagle's medium (DMEM) (3:1, 0.66 mm calcium) supplemented with 2.5% fetal calf serum, 0.4 μg/ml hydrocortisone, 8.4 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenosine, 10 ng/ml EGF, 100 units of penicillin, and 100 μg/ml streptomycin. Twenty-four hours prior to treatment, 3T3 feeder cells were removed from keratinocyte cultures by 0.5 mm EDTA rinses, and standard keratinocyte growth medium was replaced. Normal keratinocytes were not used beyond passage 5. NRK cells, clone 49F (American Type Tissue Collection, CRL 1570), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Experiments were performed with NRK cells before passage 25. All cultures were maintained at 37 °C in a 5% CO2 atmosphere.

**Cell Treatments**—For suspension studies, adherent cells were suspended in semisolid medium or treated with medium alone for the indicated times. Medium was made semisolid with sterile methylcellulose (4000 centipoises, Fisher Scientific) as described previously (Sadek and Allen-Hoffmann, 1994). Unless otherwise indicated, semisolid media were composed of serum-free Ham's F12/DMEM (3:1) containing 1.68% methylcellulose and supplemented with the aforementioned additives.

Cells were suspended following removal from tissue culture plates with 0.5 mm EDTA, 0.1% trypsin, washed with serum-containing medium to inactivate residual trypsin, then with serum-free medium, and suspended in the desired semisolid medium at a density of 1 × 10⁶ cells/ml in sterile 50-ml polypropylene tubes. Suspended cells were incubated at 37 °C in a humidified 5% CO2 atmosphere. Cells were recovered from suspension at various times by repeated dilution of the semisolid medium with serum-free medium followed by centrifugation at 440 × g. Adherent control cultures were treated with medium alone for the indicated times.

**Measurement of Internucleosomal Fragmentation**—Following treatments, 2.5 × 10⁶ cells were lysed in 500 μl of 10 mm Tris, 10 mm EDTA, pH 8.0, and 0.5% (w/v) sodium lauryl sarcosine, and the lysate was aliquoted and stored at −85 °C. Following addition of 500 μl of 10 mm Tris, 1 mm EDTA, pH 8.0 (TE buffer) the lysate was extracted twice in a siliconized microcentrifuge tube with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), treated with DNsase-free RNase A (Sigma), 50 μg/ml final concentration, for 15 min at 57 °C, and extracted again with an equal volume phenol:chloroform:isoamyl alcohol. The aqueous phase was extracted twice with an equal volume of chloroform, and nucleic acids were precipitated overnight at −85 °C following addition of 0.1 volume of 3× sodium acetate, pH 5.2, and 2 volumes of ice-cold absolute ethanol. DNA was collected by centrifugation at 16,000 × g in a Microfuge (Beckman Instruments) for 30 min. The DNA was rinsed with 95% ethanol, air-dried for 5 min, and dried in a HetoVac CS1 vacuum concentrator (InterMed, Denmark) for 5 min. The DNA was dissolved in 20 μl of TE buffer, pH 8.0. DNA was quantitated by absorption at 260 nm and stored at 4 °C at a concentration of 0.5 mg/ml in TE.

Intact and fragmented DNA was 3′-end-labeled with [α-32P]dATP using terminal deoxynucleotidyl transferase as described by Tilly.
and Hsu (1993). Briefly, each reaction mixture contained 1\times reaction buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml bovine serum albumin, pH 6.6), 2.5 mM CoCl\(_2\), 25 units of terminal deoxynucleotidyltransferase (Boehringer Mannheim), and [\(-32\)P]-ddATP (specific activity of 3000 Ci/mmol, Amersham Corp.) diluted with cold ddATP to a final concentration of 340 pM ddATP or 12.5 M\(_{c}\)GdATP. Each reaction was initiated by the addition of 360 ng of sample DNA, incubated for 60 min at 37 °C, and terminated by the addition of 25 mM EDTA, pH 8.0, final concentration. Labeled DNA was separated from unincorporated [\(-32\)P]-ddATP by precipitation with ice-cold 100% ethanol in the presence of 2 M ammonium acetate and 50 µg of yeast tRNA (Sigma) followed by a second precipitation with ice-cold 100% ethanol. The pellet was collected by centrifugation, rinsed in ice-cold 80% ethanol, dissolved in 50 µl of TE buffer, pH 8.0, and stored at −20 °C. One half of each labeled sample was loaded onto a 1.5% agarose gel and electrophoresed. Gels were dried without heat using a vacuum dryer (Bio-Rad) or with heat using a SE 1200 Easy Breeze (Hoeffer Scientific, San Francisco, CA) and exposed to X-Omat photographic film.

**Northern Analysis—Poly(A)\(^{+}\)** RNA was isolated from logarithmically growing cells as described previously (Sadek and Allen-Hoffmann, 1994). Poly(A)\(^{+}\) RNA was electrophoresed in a 1.2% agarose gel containing formaldehyde and electroblotted to a Zeta-probe membrane (Bio-Rad). The membrane was prehybridized and then hybridized in the presence of a random primer [\(^{32}\)P]-GCT-labeled cDNA probe as recommended by the supplier. The cDNA probes used were to rat glyceraldehyde-3-phosphate dehydrogenase, pGPDN5 (Fort et al., 1985), monkey TGF-\(\beta\)1 (Sharples et al., 1987), and a 830-base pair 5' fragment of human c-myc (Miyamoto et al., 1985).

**Anchorage-independent Growth Assay—** Anchorage-independent growth of NRK-49F cells in keratinocyte-conditioned semisolid medium was determined by a methylcellulose/agar assay. Conditioned semisolid medium was generated by the suspension of subconfluent cultures of keratinocytes in serum-free Ham’s F12/DMEM (3:1) supplemented with 1.68% methylcellulose and antibiotics for the indicated times. Keratinocytes were suspended in serum-free and additive-free semisolid medium and immediately removed (t = 0). Anchorage-independent growth of NRK-49F cells was used as a bioassay to determine the presence of biologically active TGF-\(\beta\)1 in keratinocyte-conditioned medium. Neutralizing anti-TGF-\(\beta\)1 antibody was added to the keratinocyte-conditioned medium to confirm that TGF-\(\beta\)1 alone was inducing anchorage-independent growth. As seen in Fig. 2, keratinocyte-conditioned medium supports the anchorage-independent growth of the NRK-49F indicator cells. Surprisingly, non-acidified keratinocyte-conditioned medium supported NRK-49F colony formation as well as acidified medium (data not shown). These results demonstrate that the number of anchorage-independent colonies is proportional to the amount of time the keratinocytes conditioned the semisolid medium. Suspended keratinocytes typically produced the equivalent of between 50 and 100 pg of recombinant human TGF-\(\beta\)1/ml of semisolid medium. Inhibition of TGF-\(\beta\)1 with neutralizing anti-TGF-\(\beta\)1 antibody decreased NRK-49F colony formation to background levels. Twice as much antibody was required to neutralize the activity in 48-h keratinocyte-conditioned medium. Therefore, loss of keratinocyte cell-cell and/or cell-substratum interaction is an inducer of TGF-\(\beta\)1 gene expression and ultimately promotes increased synthesis and perhaps activation of this epithelial growth inhibitor.

**Results**

**Suspension of Keratinocytes Induces TGF-\(\beta\)1 Expression—** In certain simple epithelial cell types and hematopoietic cells, TGF-\(\beta\)1 has been reported to be induced programmatically cell death (Oberhammer et al., 1992; Rotello et al., 1991; Lotem and Sachs, 1992). Because suspension is a potent inducer of apoptosis in normal human keratinocytes, we asked if suspension affected the level of autocrine production of keratinocyte TGF-\(\beta\)1. Poly(A)\(^{+}\) RNA was isolated from adherent keratinocytes and cells suspended in serum-free semisolid medium for 6, 12, 24, and 72 h. Two keratinocyte strains from different donors were analyzed. In both strains suspension caused an increase in the steady state levels of TGF-\(\beta\)1 mRNA within 6 h (Fig. 1). Concomitant with the increase in keratinocyte TGF-\(\beta\)1 mRNA levels, steady state c-myc RNA levels decline in a time-dependent manner. This finding is consistent with the known effects of TGF-\(\beta\)1 on c-myc gene expression in keratinocytes. These results suggest that suspension increases the amount of TGF-\(\beta\)1 protein produced by keratinocytes.

We next determined if suspended keratinocytes produced increased levels of biologically active TGF-\(\beta\)1. Keratinocytes were suspended in serum-free and additive-free semisolid medium for 0, 12, 24, and 48 h. Following suspension, keratinocytes were removed by centrifugation to generate keratinocyte-conditioned semisolid medium. To control for lysis of keratinocytes following centrifugation, keratinocytes were suspended in semisolid medium and immediately removed (t = 0). Anchorage-independent growth of NRK-49F cells was used as a bioassay to determine the presence of biologically active TGF-\(\beta\)1 in keratinocyte-conditioned medium. Neutralizing anti-TGF-\(\beta\)1 antibody was added to the keratinocyte-conditioned medium to confirm that TGF-\(\beta\)1 alone was inducing anchorage-independent growth. As seen in Fig. 2, keratinocyte-conditioned medium supports the anchorage-independent growth of the NRK-49F indicator cells. Additionally, non-acidified keratinocyte-conditioned medium supported NRK-49F colony formation as well as acidified medium (data not shown). These results demonstrate that the number of anchorage-independent colonies is proportional to the amount of time the keratinocytes conditioned the semisolid medium. Suspended keratinocytes typically produced the equivalent of between 50 and 100 pg of recombinant human TGF-\(\beta\)1/ml of semisolid medium. Inhibition of TGF-\(\beta\)1 with neutralizing anti-TGF-\(\beta\)1 antibody decreased NRK-49F colony formation to background levels. Twice as much antibody was required to neutralize the activity in 48-h keratinocyte-conditioned medium. Therefore, loss of keratinocyte cell-cell and/or cell-substratum interaction is an inducer of TGF-\(\beta\)1 gene expression and ultimately promotes increased synthesis and perhaps activation of this epithelial growth inhibitor.
were analyzed for nucleosomal fragmentation and the ability to initiate a colony when replated in surface culture. In adherent keratinocytes, treatment with TGF-β1 for 24 h did not alter the colony-forming ability upon replating when compared with control (Fig. 3A) or induce DNA fragmentation (Fig. 3B, compare lanes 1 and 2). Surprisingly, treatment of suspended keratinocytes with TGF-β1 attenuated suspension-induced DNA fragmentation (Fig. 3B, compare lanes 3 and 4). Furthermore, addition of TGF-β1 to the semisolid medium modestly enhanced the colony-forming potential of suspended keratinocytes. We next determined if endogenous TGF-β1 from suspended keratinocytes affected DNA fragmentation. To inhibit the effects of suspension-mediated endogenous TGF-β1, we suspended keratinocytes in semisolid medium containing a TGF-β1 neutralizing antibody. Neutralization of keratinocyte TGF-β1 consistently increased DNA fragmentation following suspension (Fig. 4), although the magnitude of this response varies among experiments. Therefore, in normal human keratinocytes, TGF-β1 appears to attenuate nucleosomal fragmentation following abrogation of cell-substratum and/or cell-cell interactions.

**DISCUSSION**

We found that suspended keratinocytes contained increased levels of both TGF-β1 mRNA and activated protein. TGF-β1 is an autocrine growth factor that inhibits the growth of adherent human keratinocytes in vitro (Shipley et al., 1986; Pietenpol et al., 1990). Suspension of keratinocytes also results in a decrease in the steady state mRNA levels of c-myc. This finding is consistent with the known effects of suspension and TGF-β1 on the growth of keratinocytes. We suspect that the suspension-induced decrease in c-myc mRNA levels may be mediated, in part, through increased production of TGF-β1. The role of TGF-β1 in terminal differentiation or programmed cell death in stratified squamous epithelia is unclear. In vivo, TGF-β1 plays a role in the maintenance of normal tissue architecture in rodent skin. For example, transgenic mice homozygous for a targeted disruption of the TGF-β1 locus exhibit epidermal hyperproliferation as measured by an increase in the labeling index of basal cell nuclei following administration of 5-bromo-2'-deoxyuridine (Glick et al., 1993). However, these TGF-β1 null mice express the differentiation-specific keratins, K1 and K10, normally (Glick et al., 1993). Based on these reports and our findings it is reasonable to postulate that TGF-β1 may exert its effect on epidermal homeostasis by affecting the num-

**FIG. 2.** Conditioned medium from suspended keratinocytes contains active TGF-β1. Cultured keratinocytes were suspended in serum-free, additive-free, semisolid growth medium for 12, 24, and 48 h to generate conditioned semisolid medium (CM). Keratinocytes were removed from the conditioned medium by centrifugation without dilution of the semisolid medium. To control for possible cell lysis during centrifugation, keratinocytes were suspended in semisolid medium and immediately removed by centrifugation (0 h conditioned semisolid medium). Indicator cells, NRK-49F, were assayed for anchorage-independent growth in the keratinocyte-conditioned media. Conditioned medium was incubated with or without a neutralizing anti-human TGF-β1 antibody (2.5 µg/ml) for 1 h prior to the addition of NRK-49F cells. As a control for background levels of anchorage-independent growth, NRK-49F cells were suspended in semisolid medium supplemented with EGF alone. A standard curve for TGF-β1-dependent anchorage-independent NRK-49F cell growth was generated for each experiment using the following concentrations of recombinant TGF-β1: 0.05, 0.1, 0.5, 1.0, and 2.0 ng/ml semisolid medium. After 7 days, anchorage-independent colonies of NRK-49F cells were scored. Data represent the average number (mean ± S.D. of three replicates per condition) of colonies (>60 µm) per well.

**FIG. 3.** TGF-β1 inhibits internucleosomal cleavage in suspended keratinocytes. Keratinocytes (NS-2-Ep) were suspended for 24 h in serum-free, semisolid growth medium containing 5 ng/ml TGF-β1 or an equal volume of diluent (4 mM HCl, 1 mg/ml bovine serum albumin). Adherent control cells were refed with serum-free growth medium containing 5 ng/ml TGF-β1 or diluent at the beginning of the treatment period. A, keratinocytes were plated onto mitomycin C-treated 3T3 feeder layers to determine the effect of treatments on replicative potential in surface culture. After 14 days, cultures were fixed with formaldehyde, stained with methylene blue, and counted. Data represent the average colony-forming efficiency (CFE, mean ± S.D. of six replicates per treatment) for each condition. B, following treatment, genomic DNA was isolated, labeled on 32P-ddATP, displayed on a 1.5% agarose gel, and subjected to autoradiography.

**FIG. 4.** Neutralization of keratinocyte-derived TGF-β1 promotes internucleosomal cleavage in suspended keratinocytes. Keratinocytes (NS-2-Ep) were suspended for 24 h in serum-free semisolid growth medium in the presence or absence of a neutralizing antibody (Ab) to human TGF-β1. Adherent control cells received serum-free growth medium with or without anti-TGF-β1 antibody at the beginning of the 24-h treatment period. Following treatments, genomic DNA was isolated, labeled on 3'-ends with [α-32P]dATP, displayed on a 1.5% agarose gel, and subjected to autoradiography.
number of cells present in the tissue as opposed to directly affecting the synthesis and/or organization of differentiation-specific proteins.

We have recently found that suspension of normal human keratinocytes induces both morphological and biochemical features of programmed cell death, such as membrane blebbing and DNA fragmentation. Because TGF-β1 has been implicated in the regulation of programmed cell death in a variety of epithelial cell types (Rotello et al., 1991; Oberhammer et al., 1991; Bursch et al., 1993), we determined if endogenous production of TGF-β1 played a role in suspension-mediated apoptotic signaling in keratinocytes. In contrast to findings in hepatocytes and hematopoietic and lymphoid cells, we found that TGF-β1 protected against suspension-induced DNA fragmentation in keratinocytes. Treatment of suspended keratinocytes with exogenously added TGF-β1 dramatically attenuated suspension-induced nucleosomal fragmentation. Furthermore, neutralizing endogenously produced TGF-β1 activity in suspended keratinocytes increased DNA fragmentation. Neither DNA fragmentation nor colony-forming efficiency was affected by treatment of adherent cells with TGF-β1. It is possible that TGF-β1 delays the onset of suspension-induced DNA fragmentation rather than blocking the apoptotic response altogether. Nevertheless, it is clear that TGF-β1 does not promote apoptosis in adherent or suspended human keratinocytes in vitro. Based on these findings, we propose that TGF-β1 functions to protect cultured keratinocytes from suspension-induced DNA fragmentation. Consistent with this interpretation, TGF-β1 is present exclusively in the basal layer of normal mouse epidermis (Glick et al., 1993) where it may serve to protect against apoptosis in the replicative compartment of skin. TGF-β1 also increases expression of cell adhesion molecules such as fibronectin, collagens, and their cognate cell surface receptors (reviewed by Massague (1990)), thereby potentially increasing cellular adhesiveness. Our findings may have important implications for the current understanding of normal epidermal wound healing (reviewed by Gailit and Clark (1994)). For example, increased endogenous levels of TGF-β1, as well as that contributed by platelets, may serve to forestall injury-induced apoptosis in a wound while increasing the production of extracellular matrix and keratinocyte adhesiveness. TGF-β1, although a potent keratinocyte growth inhibitor, may serve to forestall apoptosis in this renewal tissue during times of tissue regeneration.

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