The transforming growth factor-β (TGF-β) family consists of an increasing number of related, but functionally distinct proteins (1, 2). One member of this family, TGF-β1, is produced and secreted as a latent, high molecular weight complex by many normal and neoplastic cells (1–3). TGF-β1 is synthesized as a 390-amino acid precursor polypeptide which is both glycosylated and phosphorylated (4, 5). Proteolytic cleavage at the carboxyl terminus yields the mature 112-amino acid TGF-β1 monomer; active TGF-β1 consists of two identical monomer units linked by disulfide bridges (2). The complete amino acid sequence of normal and mouse TGF-β1, which are deduced from their cDNA sequences, are remarkably homologous (6, 7). TGF-β2 has 72% amino acid homology with TGF-β1 and induces many of the same effects (2).

TGF-β1 and TGF-β2 regulate cellular proliferation, differentiation, and other functions in many cell types (1, 2). They can inhibit as well as stimulate cell proliferation, depending on the cell line/type and the growth conditions (8–12). TGF-β1 is a promoter of chondrogenesis and a stimulator of angiogenesis (13, 14). In tracheobronchial epithelial cells TGF-β1 induces terminal cell division and expression of the squamous differentiated phenotype (15, 16). However, TGF-β1 inhibits adipogenic and myogenic differentiation (17, 18). In NHEK cells, TGF-β1 induces reversible growth arrest (19, 20). Many mesenchymal and epithelial cells which are affected by TGF-β1 exhibit elevated expression of several extracellular matrix components and protease inhibitors (14, 21–25). The cellular responses to TGF-β1 and TGF-β2 are mediated by specific cell-surface receptors (2, 26). However, little is known about the signal transduction mechanism through which TGF-β acts.

Our laboratory has been interested in the regulatory factors that modulate proliferation and differentiation of NHEK cells (27, 28). In studies designed to examine the effect of TGF-β1 on these processes we found that TGF-β1 enhanced the level of transglutaminase activity in these cells. Transglutaminases (R-glutaminylpeptide:amine-γ-glutamyltransferase, EC 2.3.2.13) form a family of distinct, Ca²⁺-dependent enzymes that catalyze the formation of ε-(γ-glutamyl)lysine-protein cross-links (29). Plasma factor XIIIa, a transglutaminase formed from the zymogen Factor XIII, catalyses the polymerization of fibrin. A soluble transglutaminase, termed tissue or Type II transglutaminase, has been identified in many cell types; its function has still to be determined (30, 31). Epidermal or Type I transglutaminase is membrane-bound and catalyzes the formation of cross-linked envelopes during squamous cell differentiation (32–34). Recently, cDNA's coding for Type II transglutaminase (35–37), plasma factor XIIIa (38), and Type I transglutaminase (39) have been isolated and sequenced. In this paper, we demonstrate that TGF-β1 and TGF-β2 regulate specifically the expression of transglutaminase Type II.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Second passage cultures of normal epidermal keratinocytes isolated from human foreskin were obtained from Clonetics...
Corp., San Diego, CA. Cells were grown in modified MCDB153 medium containing 0.15 mM CaCl₂ and supplemented with 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 1.4 mM hydrocortisone, and bovine pituitary extract (50 µg/ml), as described by Boyce and Ham (40). Complete medium without bovine pituitary extract (KDM) and with bovine pituitary extract (KGM) were obtained from Clonetics (Harrington, MA) and from Dr. J. McLane (Hoffmann-La Roche, Nutley, NJ), respectively. The cell line SCC-15 was obtained from ATCC (Rockville, MD). Culture media were obtained from R&D Systems Inc., Minneapolis, MN. NHEK cells were treated with TGF-β1 and TGF-β2 in the exponential growth phase, unless otherwise stated. Squamous differentiated cells were obtained by maintaining cultures for several days at confluence, as described previously (27, 28).

Transglutaminase Assays—Cells grown in 60-mm dishes were washed in ice-cold phosphate-buffered saline containing 1 mM CaCl₂ and 1 mM sodium fluoride. Cells were detached by three freeze and thaw cycles. The homogenate was centrifuged at 105,000 × g, yielding the particulate and soluble fractions. Transglutaminase assays were performed on the total homogenate to determine total transglutaminase activity and on the particulate and soluble fractions to determine Type I (epidermal) and Type II (tissue) transglutaminase, respectively. Transglutaminase activity was measured by determining the incorporation of [³H]putrescine (16.2 Ci/mmol; Du Pont, Boston, MA) into casein hydrolysate as described previously (34), and was expressed as disintegrations/min of [³H]putrescine incorporated per mg of total cellular protein.

Cholesterol Sulfate Determination—Cells were incubated for 24 h in medium containing 25 µCi/ml Na₂³²SO₄ (carrier-free; ICN, Irvine, CA). Cells were harvested by trypsinization, pelleted by centrifugation, and extracted with 4 ml of chloroform/methanol as described previously (34). A small reduction in colony-forming efficiency in agreement with previous observations (27, 32), cultures of undifferentiated NHEK cells exhibited, throughout the exponential growth phase, low levels of total transglutaminase activity (about 4 dpm/h/µg of protein). About 50% of the total transglutaminase activity was associated with the particulate fraction, representing epidermal or Type I transglutaminase, whereas the other 50% was associated with the soluble fraction representing tissue or Type II transglutaminase (27, 32). Confluent cultures of differentiated NHEK cells contained a 15-fold higher level of transglutaminase activity that was largely (95%) associated with the particulate fraction, consistent with the increase in transglutaminase Type I during squamous differentiation of NHEK cells (27, 32-34).

When logarithmic cultures of NHEK cells were treated with 100 µM TGF-β1 for 2 days, a 7-fold stimulation in total cell-associated transglutaminase activity was observed (Table I). This increase in activity was due solely to a 13-fold increase in the transglutaminase activity associated with the soluble fraction (Type II transglutaminase). In order to confirm the identity of the TGF-β1-stimulated transglutaminase as Type II transglutaminase, total cellular protein from NHEK cells of confluent, squamous differentiated cultures, logarithmic cultures treated for 2 days with 100 µM TGF-β1, and undifferentiated logarithmic cultures were analyzed by immunoblot analysis using monoclonal antibodies B.C1 and Cub-7401 (32, 46). These two antibodies react specifically with either Type I or Type II transglutaminase which have been shown to migrate upon SDS-polyacrylamide gel electrophoresis as monomers with molecular weights of 90,000 and 82,000, respectively (32, 30). Relatively little of either Type I or II transglutaminase could be identified in undifferentiated NHEK cells (Fig. I). The monoclonal antibody Cub-7401, which reacts specifically with Type II transglutaminase, stained a 82-kDa protein from TGF-β1-treated cells, whereas no staining was observed with the monoclonal antibody B.C1 which reacts with Type I transglutaminase. The antibody B.C1 stained specifically a 90-kDa protein from squamous differentiated cells, whereas no staining was observed with the antibody Cub-7401. These results confirm that TGF-β1-treated cells express mostly transglutaminase Type II. In contrast, squamous differentiated cells contain mostly transglutaminase Type I in agreement with previous findings (28, 32).

The lack of induction of Type I transglutaminase by TGF-β1 suggests that TGF-β1 does not induce squamous differentiation in NHEK cells. Additionally, TGF-β1 did not induce cholesterol sulfate (Table I), another biochemical marker of squamous cell differentiation (49). TGF-β1 caused only a small reduction in colony-forming efficiency in agreement with previous studies (19, 20) demonstrating reversible growth arrest by TGF-β1 in epidermal keratinocytes. High calcium concentrations in the medium (2 mM instead of 0.15 mM) also did not induce a squamous differentiated phenotype in TGF-

Regulation of Transglutaminase Activity

Preparation of Northern Blots—RNA was electrophoresed through a 1% agarose-formaldehyde gel, ribosomal RNA and Bethesda Research Laboratories RNA ladder were used as size markers. Following electrophoresis, the RNA was transferred for partial alkaline hydrolysis and subsequent neutralization of the gel (48), and the RNA was transferred to GeneScreen membrane (Du Pont) or Nytran by capillary action using 1.5 M NaCl, 0.15 M sodium citrate, pH 7 (10 × SSC), as the transfer buffer. Following transfer, the RNA was cross-linked to the blot by UV irradiation.

Hybridization of Northern Blots—Northern blots were prehybridized for 4-24 h at 42 °C in a buffer containing 50% formamide, 5 × SSPE, 2 × Denhardt's 1% SDS, and 250 µg/ml sheared salmon sperm DNA. Following addition of the probe (5-15 µg/ml), hybridization was allowed to proceed overnight at 42 °C. Blots were washed at a final stringency of 60 °C in 0.1 × SSC, 0.1% SDS.

RESULTS

In agreement with previous observations (27, 32), cultures of undifferentiated NHEK cells exhibited, throughout the exponential growth phase, low levels of total transglutaminase activity (about 4 dpm/h/µg of protein). About 50% of the total transglutaminase activity was associated with the particulate fraction, representing epidermal or Type I transglutaminase, whereas the other 50% was associated with the soluble fraction representing tissue or Type II transglutaminase (27, 32). Confluent cultures of differentiated NHEK cells contained a 15-fold higher level of transglutaminase activity that was largely (95%) associated with the particulate fraction, consistent with the increase in transglutaminase Type I during squamous differentiation of NHEK cells (27, 32-34).

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TABLE I

| Cells                      | Transglutaminase activity | Cholesterol sulfate | Colony-forming efficiency |
|----------------------------|----------------------------|---------------------|---------------------------|
|                            | Total                      | Soluble             | Particulate               | dpm/µg protein | %     |
| Undifferentiated           | 0.4                        | 0.2                 | 0.2                       | 3.0 ± 0.5     | 38 ± 5 |
| Squamous differentiated     | 6.4                        | 0.1                 | 6.3                       | 96.0 ± 6.0    | <0.1  |
| TGF-β1-treated             | 2.9                        | 2.6                 | 0.3                       | 3.0 ± 0.6     | 29 ± 6 |

Fig. 1. Expression of Type I and Type II transglutaminase in NHEK cells. Cells grown under different conditions were solubilized and cellular proteins separated by SDS-polyacrylamide gel electrophoresis and examined via immunoblot analysis using two monoclonal antibodies, B.C1 and Cub-7401, specific for Type I or Type II transglutaminase, respectively. 1, NHEK cells grown in the exponential phase; 2, NHEK cells treated in the exponential phase with 100 pM TGF-β1 for 48 h; 3, confluent, squamous differentiated NHEK. Molecular weight markers are indicated.

Fig. 2. Stimulation of transglutaminase activity in NHEK cells by TGF-β1. NHEK cells were plated in 60-mm dishes at 5.0 x 10⁴ cells/dish and 2 days later incubated in the presence or absence of TGF-β1 (100 pM). At different time intervals after the addition of TGF-β1, transglutaminase activity was determined. A and B, undifferentiated cells; C, squamous differentiated NHEK cells. Squares, total transglutaminase activity associated with cells; triangles, transglutaminase activity associated with the particulate fraction; circles, transglutaminase activity associated with the soluble fraction. All transglutaminase activities were calculated as per mg of total cellular protein.

The increase in total and Type II transglutaminase activity was first measurable 8 h after the addition of TGF-β1 and reached a plateau after 3 days of treatment (Fig. 2B). Exposure of undifferentiated NHEK cells to 100 pM TGF-β1 for 3 days stimulated Type II transglutaminase activity about 15-fold; little effect was observed on the level of the transglutaminase activity associated with the particulate fraction (Type I transglutaminase). No significant changes in the level of transglutaminase activity were observed in untreated cells over this incubation period (Fig. 2A). Moreover, no increase in transglutaminase Type II was observed when confluent cultures of differentiated NHEK cells were exposed to TGF-β1 (Fig. 2C). These results show that undifferentiated and squamous differentiated NHEK cells respond differentially to TGF-β1. No transglutaminase activity was detectable in the medium from cultures of either untreated or TGF-β1-treated NHEK cells (not shown). The increase in transglutaminase Type II activity was obtained in KGM as well as KDM medium and was observed consistently in epidermal cells isolated from human neonatal foreskin or human adult breast skin of different donors (not shown).

The increase in transglutaminase Type II activity was dependent on the TGF-β1 concentration (Fig. 3). Half-maximum stimulation occurred at a concentration of approximately 15 pm TGF-β1. The stimulation was optimal between 30 and 100 pm TGF-β1. TGF-β2 was just as effective as TGF-β1 in either inhibiting proliferation of NHEK cells or inducing Type II transglutaminase activity (Fig. 4). The half-maximum concentration of TGF-β to inhibit proliferation was 1.5 pm, about 10-fold lower than for the induction of transglutaminase Type II. The dose-response was further examined via immunoblot analysis (Fig. 5). NHEK cells growing in the early exponential phase were treated with various concentrations of TGF-β1 for 3 days, then solubilized in SDS sample buffer and total cellular proteins examined by immunoblot analysis using the transglutaminase Type II-specific monoclonal antibody Cub-7401. The staining of a 80-kDa protein band, representing the Type II transglutaminase, was enhanced.
Regulation of Transglutaminase Type II

FIG. 3. Effect of TGF-β1 on transglutaminase activity. NHEK cells were treated with TGF-β1 at the indicated concentration. Three days after TGF-β1 treatment, cells were assayed for transglutaminase activity. ○, total transglutaminase activity; Δ, transglutaminase activity associated with particulate fraction; △, transglutaminase activity in soluble fraction.

FIG. 4. Comparison of the action of TGF-β1 and TGF-β2 on cell proliferation and Type II transglutaminase activity. NHEK cells grown in early exponential phase were treated with the indicated concentration of TGF-β1 (○) or TGF-β2 (●). Cell numbers were determined after 5 days of treatment (A). Transglutaminase Type II activity was assayed after 3 days of treatment (B).

with increasing concentrations of TGF-β1. Densitometric analyses indicated that 100 pM TGF-β1 caused a 10- to 15-fold increase in transglutaminase Type II protein.

FIG. 5. Dose-response of the increase in Type II transglutaminase by TGF-β1. NHEK cells were treated with various concentrations of TGF-β1. After 3 days of treatment, cells were solubilized and cellular proteins separated by SDS-polyacrylamide gel electrophoresis. A, Coomassie Brilliant Blue staining. B, immunoblot using monoclonal antibody Cub-7401 against transglutaminase Type II. Lane 1, untreated cells; lane 2, 3 pM TGF-β1; lane 3, 10 pM; lane 4, 30 pM; lane 5, 100 pM. Molecular weight markers (kD) are indicated. Arrow marks the position of the 82-kDa band reacting specifically with the monoclonal antibody Cub-7401.

FIG. 6. Differential expression of Type I and II transglutaminase mRNA in NHEK cells. Poly(A)+ RNA (3 μg) isolated from undifferentiated (exponential phase) NHEK cells (U), squamous differentiated cells (S), and TGF-β1-treated cells (T) was fractionated on a agarose-formaldehyde gel and after blotting to GeneScreen hybridized to 32P-labeled pTG-7, encoding the Type I transglutaminase (A), to 32P-labeled pTG3400, encoding Type II transglutaminase (B), and to 32P-labeled pGAD-28, encoding glyceraldehyde-3-phosphate dehydrogenase (C). The same blot was stripped and reprobed each time.

FIG. 7. Kinetics of the accumulation of Type II transglutaminase mRNA. The single 4.4-kb band representing Type II transglutaminase mRNA increased 4 h after the addition of TGF-β1 and the increase reached an optimum after 18 h. Transglutaminase Type II mRNA was undetectable in samples from untreated cells. To examine whether the TGF-β1-induced increase in Type II transglutaminase mRNA was dependent on protein synthesis, the effect of cycloheximide was studied. Two independent experiments showed that cycloheximide...
Procedures. Equivalent amounts of RNA (10 pg) were probed with

\[ ^{32}P \text{-labeled pTG3400 following agarose gel electrophoresis and trans-} \]

\[ \text{fer to Nytran. After stripping, the blot was reprobed with } ^{32}P \text{-labeled} \]

\[ \text{pGAD-28.} \]

mRNA accumulation.

We compared the action of TGF-\( \beta \)1 on transglutaminase activity in normal epidermal keratinocytes with its effect on three human squamous carcinoma cell lines (SCC-13, SCC-15, and SQCC/Y1) and two cell lines, NHEK-SV40-T8 and -T11, which express the SV40 large T-antigen. The carcinoma-derived cell lines fail to undergo squamous differentiation in culture, whereas the SV40 T-antigen-transfected cells have an extended life span and are able to undergo squamous differentiation (27). NHEK-SV40-T8 and -T11 cells did not contain any detectable levels of transglutaminase Type II activity. Treatment of these cells with TGF-\( \beta \)1 induced Type II activity more than 150-fold (Table II). The levels of Type II transglutaminase in TGF-\( \beta \)1-treated NHEK-SV40-T8 and -T11 were comparable to that of TGF-\( \beta \)1-treated NHEK cells. The larger apparent increase in the SV40 T-antigen-transformed cells was due to the presence of lower levels of this transglutaminase in untreated transformed cells. The carcinoma-derived cell lines SCC-13, SCC-15, and SCC/Y1 were much less responsive to TGF-\( \beta \)1 (Table II) than the NHEK or SV40-T-transformed cells.

**DISCUSSION**

We have been interested in those hormonal factors that regulate the growth and differentiation of normal human epidermal keratinocytes. TGF-\( \beta \)1 is one factor that has important effects on the proliferation and differentiation of many types of cells including NHEK cells (2, 19, 20). NHEK cells in culture exhibit a high proliferative capacity (high colony-forming efficiency) during the exponential growth phase. At confluence, these cells undergo irreversible growth arrest (terminal cell division) and start to express a squamous differentiated phenotype (27). This induction of differentiation is characterized by an increase in the expression of several biochemical markers that include cholesterol sulfate, transglutaminase Type I, and specific keratins (27, 32, 50). During the exponential phase of cell growth, NHEK cells express low levels of both Type I (epidermal) transglutaminase, which is associated with the particulate fraction, and Type II (tissue) transglutaminase, which is localized in the cytosol. These two transglutaminases are distinct enzymes exhibiting different immunological properties and represent two different gene products (30-34, 36, 97). Treatment of NHEK cells with TGF-\( \beta \)1 does not induce irreversible growth arrest or expression of a squamous differentiated phenotype as indicated by the retention of a relatively high colony-forming efficiency, and the low levels of expression of the differentiation markers transglutaminase Type I and cholesterol sulfate. These find-

**Table II**

**Effect of TGF-\( \beta \)1 on transglutaminase activity in transformed NHEK cells**

| Cells              | Control cells | TGF-\( \beta \)-treated cells* |
|--------------------|---------------|-------------------------------|
|                    | Total          | Soluble                       | Total          | Soluble         |
|                    | dpm/h/mg protein \( \times 10^9 \) |                          | dpm/h/mg protein \( \times 10^9 \) |
| NHEK-SV40-T8       | 2.0 ± 0.2      | 1.2 ± 0.2                     | 2.0 ± 0.2      | 9.5 ± 4.4       |
| NHEK-SV40-T11      | 3.0 ± 0.2      | <0.1                          | 1.4 ± 0.1      | 15.1 ± 0.5      |
| SCC-13             | 3.4 ± 0.2      | 0.8 ± 0.3                     | 3.4 ± 0.2      | 14.9 ± 0.6      |
| SCC-15             | 4.4 ± 0.2      | 0.7 ± 0.2                     | 4.4 ± 0.2      | 14.9 ± 0.6      |
| SQCC/Y1            | 2.1 ± 0.2      | 0.9 ± 0.2                     | 2.1 ± 0.2      | 0.5 ± 0.2       |

* Cells in the exponential phase were treated for 2 days with 100 pm TGF-\( \beta \)1 and then assayed for transglutaminase activity.

(2.5 \( \mu \)g/ml) abrogated the increase in Type II transglutaminase mRNA by TGF-\( \beta \)1 (Fig. 9) indicating that the increase in transglutaminase Type II mRNA is dependent on protein synthesis. In contrast, glyceraldehyde-3-phosphate dehydrogenase mRNA expression was unaffected by either TGF-\( \beta \)1 or cycloheximide.
ings are in agreement with previous studies showing that TGF-β does not induce terminal differentiation in NHEK cells (19, 20). However, addition of TGF-β1 or TGF-β2 results in a 10- to 15-fold stimulation of transglutaminase Type II activity. This enhancement in transglutaminase activity appears to be related to increased synthesis of the transglutaminase Type II enzyme and to increased levels of corresponding mRNA. The increase in mRNA levels is cycloheximide sensitive indicating that protein synthesis is required for this action.

TGF-β1 has been shown to stimulate the synthesis of a number of gene products, such as fibronectin, collagen, and TGF-β1 itself (2, 21, 22, 24, 51, 52), in several cell systems. In NHEK cells, TGF-β1 also induces the synthesis of fibronectin and to a lesser degree the synthesis of collagen Type IV. TGF-β1 can regulate gene expression at the level of transcription as well as at the level of the stability of the mRNA (22–24, 51). Like the increase in Type II transglutaminase mRNA, the stimulation of both fibronectin and collagen α2(1) mRNA by TGF-β1 have been shown to depend on protein synthesis. Binding sites for the transcriptional factor nuclear factor 1 in the promoter regions of the collagen α2(1) and fibronectin gene have been implicated in the regulation of these genes by TGF-β1 (24, 51). However, other elements appear to be involved in the TGF-β1 inducibility as well (24). Although the full-length coding sequence of human and guinea pig transglutaminase Type II mRNA’s have been established, no data are as yet available about the promoter sequence (35, 36). Efforts are under way to sequence the promoter region of human Type II transglutaminase and to determine its regulatory elements. It will be interesting to see whether nuclear factor 1-binding sites are involved in the regulation of this enzyme by TGF-β1.

TGF-β1 did not induce transglutaminase Type II activity in certain epidermal carcinoma-derived cell lines. The nonresponsiveness of these cells to TGF-β1 appears not to be due to the absence of cell surface receptors since high affinity receptors have been demonstrated in these cells (52, 53) but could be related to an alteration in the TGF-β1-induced signal transduction at a level other than the TGF-β1/receptor interaction or due to the activation of a signal that antagonizes the TGF-β response.

Various other factors have been shown to induce Type II transglutaminase in different cell systems. Sodium butyrate induces Type II transglutaminase activity in human fibroblasts W1-38 (54) and in PC12 pheochromocytoma cells (55). Retinoic acid has been shown to stimulate Type II transglutaminase in human myeloblastic leukemia HL60 cells and in mouse peritoneal macrophages (30, 31). In the latter case, the induction is further stimulated by cAMP and inhibited by pertussis toxin. Type II transglutaminase activity was not further modulated by sodium butyrate, cAMP, or pertussis toxin in either TGF-β1-treated or untreated NHEK cells, indicating differences in the mechanism of transglutaminase induction in the various cell systems. In human epidermal keratinocytes grown on 3T3 feeders and in the presence of serum, as well as in mouse epidermal cells, retinoic acid has been shown to increase Type II transglutaminase activity (33, 56); however, in serum-free medium and in the absence of feeder cells, retinoic acid was unable to induce transglutaminase Type II activity under a wide variety of conditions (27). Differences in culture conditions might account for this inconsistency. Possibly, the stimulation of transglutaminase

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11104

Regulation of Transglutaminase Type II

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