Interferon γ (IFN-γ) is a potent inducer of squamous differentiation in normal human epidermal keratinocytes. This induction is characterized by a ≥95% decrease in the mRNA level of two growth regulatory genes, cdc2 and E2F-1, and a 7–15-fold increase in the expression of two squamous cell-specific genes, transglutaminase type I and cornifin. In contrast to the decrease in cdc2 and E2F-1 expression, the increase in transglutaminase type I and cornifin mRNAs by IFN-γ occurs after a lagtime of more than 12 h. These results are consistent with the hypothesis that in normal human epidermal keratinocyte cells irreversible growth arrest precedes the expression of the squamous-differentiated phenotype. The action of IFN-γ on the expression of squamous cell-specific genes is antagonized by retinoic acid and transforming growth factor β1. Both factors are potent suppressors of the induction of transglutaminase type I and cornifin; however, they do not prevent the commitment to irreversible growth arrest.

Several squamous cell carcinoma cell lines do not show a detectable decrease in cdc2 or increase in transglutaminase type I mRNA levels after IFN-γ treatment and appear to be altered in their control of squamous differentiation.

The epidermis is a stratified squamous epithelium that consists of four histologically distinct layers (1). In normal skin, cells in the basal layer comprise the proliferative compartment (2). These cells undergo irreversible growth arrest and start to express squamous cell-specific genes when they transit into the suprabasal (spiny) layer (1–5). Similar to other cell systems (6), irreversible growth arrest in NHEK cells is closely associated with the expression of the differentiated phenotype (5). The events that link the control of growth arrest with the induction of the squamous differentiated phenotype have yet to be elucidated. The expression of squamous differentiation-specific genes is induced at very specific stages during squamous differentiation and appears to be controlled at the transcriptional level (4, 7–9). A characteristic feature of squamous differentiation is the formation of the cross-linked envelope, a layer of cross-linked protein deposited just beneath the plasma membrane (10, 11). Transglutaminase type I catalyzes the formation of ε-(γ-glutamyl)lysine linkages between envelope precursor proteins such as involucrin, cornifin, and loricrin (9, 12–18). A variety of factors including activators of protein kinase C, calcium ions, retinoids, and transforming growth factor β1 (TGF-β1) have been shown to influence epidermal differentiation (18–22).

IFN-γ, a cytokine produced by activated T-cells and natural killer cells, affects a vast array of different cellular processes, such as antiviral responses, cell growth and differentiation, and immunoregulatory functions (23, 24). Cellular responses to IFN-γ are mediated by a specific high-affinity receptor expressed at the cell surface (25–27). Binding of IFN-γ to its receptor results in activation of a specific protein kinase and the subsequent phosphorylation and activation of a latent cytoplasmic protein, γ-activated factor (28). After translocation to the nucleus, this factor interacts with a specific DNA element (γ-activated site) in the promoter of target genes such as the guanylate-binding protein resulting in an altered rate of transcription (28–30). In addition, the activation of specific phosphatase(s) and kinase(s) by IFN-γ may influence other signaling pathways such as protein kinase C (31) or sphingomyelin (32).

Evidence has been provided suggesting that IFN-γ plays a role in the regulation of proliferation and differentiation in the epidermis (33). IFN-γ has also been reported to inhibit growth and induce the production of TGF-α and intercellular adhesion molecule-1 in cultured epidermal keratinocytes (34, 35). In the present study, we analyze in more detail the action of IFN-γ on the expression of several growth regulatory and differentiation-specific genes in cultured NHEK cells and in several squamous cell carcinoma cell lines. In addition, we examine the interference of two established modulators of epidermal differentiation, retinoic acid, and TGF-β1, with the IFN-γ-induced squamous differentiation. Both retinoic acid and TGF-β1 antagonize the induction of squamous cell-specific genes; however, these factors do not affect the induction of irreversible growth arrest by IFN-γ. Our findings are consistent with the hypothesis that IFN-γ is an effective inducer of squamous cell differentiation in NHEK cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant IFN-γ and TGF-β1 were purchased from R&D, Minneapolis, MN. All-trans-retinoic acid was a gift from Hoffmann-La Roche. Retinoic acid was dissolved in dimethyl sulfoxide.

**Cell Culture**—Second passage cultures of normal epidermal keratinocytes isolated from human foreskin were obtained from Clonetics Corp. (San Diego, CA) and were grown in keratinocyte growth medium (Clonetics). The human squamous cell carcinoma cell lines SCC13, SQCC/ Y1, and SVK14 were obtained from Dr. J. G. Rheinwald (Harvard University, Boston, MA), Dr. J. McLane (Hoffmann-La Roche), and Dr. S. Michel (Centre International de Recherches Dermatologique Galderma, Sophia Antipolis, France), respectively. All cell lines were maintained in keratinocyte growth medium. To measure DNA synthesis cells were labeled with 2.5 μCi/ml [3H]thymidine (5.0 mCi/mmol, Amersham Corp.) for 3 h at 37°C. Cells were then washed in phosphate-buffered saline.
saline and incubated in 1 ml of methanol/acetic acid (9:1) for 1 h at 4 °C. Cells were then treated with 0.5 ml of 0.2 M NaOH at 37 °C for 30 min. After neutralization, aliquots were taken for the determination of protein and radioactivity. Colony forming efficiency was determined as described previously (22).

Transglutaminase Assay—Cells grown in 60-mm dishes were washed in ice-cold phosphate-buffered saline containing 1 M EDTA and 1 M phenylmethylsulfonyl fluoride. Cells were disrupted 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 the activities of type I and type II transglutaminase, respectively. Transglutaminase activity was measured by determining the incorporation of [3H]putrescine (16.2 Ci/mmol, Dupont) into casein hydrolysate (Sigma) as described previously (36), and was expressed as disintegrations/min of [3H]putrescine incorporated in 1 h/mg of total cellular protein.

cDNA Probes—Probes for E2F-1, human IFN-γ receptor (using amplimers from Clontech) and cdc2 (37) were prepared by polymerase chain reaction amplification. The cDNA probe for c-myc was purchased from Oncogene Science (Uniondale, NY). The cDNA probes for Rb and p53 were obtained from Dr. Y. Fung (University of Southern California, Los Angeles, CA) and Dr. M. Oren (Weizmann Institute of Science, Rehovot, Israel), respectively. The recombinant cDNA clones pTG-7 and pTG3400 contain fragments of the coding region of transglutaminase type I and type II, respectively (14, 20). The cDNA probe for chicken glyceraldehyde-3-dehydrogenase consisted of a 1.12-kb PstI restriction fragment of plasmid pHT-21 (39). All probes were gel purified and labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham Corp.) via random priming using the kit and protocols supplied by Bethesda Research Laboratories.

Preparation of RNA and Northern Blot Analysis—Cultured NHEK cells were solubilized in guanidinium isothiocyanate and the extract centrifuged through a discontinuous CsCl gradient to pellet the total RNA (40). Poly(A)+ RNA was obtained by affinity chromatography through oligo(dT)-cellulose push columns (Stratagene, La Jolla, CA). RNA was electrophoresed on a 1.2% agarose-formaldehyde gel. Following electrophoresis, RNA was transferred to Nytran (Schleicher & Schuell) and then cross-linked by UV irradiation. Northern blots were prehybridized for 24 h at 42 °C in a buffer containing 50% Formamide, 2× SSPE, 2× Denhardt's, 1% SDS, and 250 μg/ml sheared salmon sperm DNA as described (40). Following addition of the labeled probe (3×15 ng/ml), hybridization was allowed to proceed overnight at 42 °C. Blots were washed at a final stringency of 60 °C in 0.2× SSC, 0.1% SDS. Blots were exposed to Kodak XAR5 film and quantitated by scanning laser densitometry using the GSXL2 software package (Pharmacia LKB Biotechnology Inc.).

Immunoblot Analysis—Immunoblot analysis using an anti-cornifin antiserum (SQ37A-Ab) was performed as described previously (18).

RESULTS

To study the action of IFN-γ on squamous cell differentiation in NHEK cells, we examined its effect on two stages of this differentiation process, irreversible growth arrest and expression of the squamous phenotype. IFN-γ inhibited the proliferation of NHEK cells in a concentration-dependent manner (Fig. 1A). The concentration of half-maximum inhibition (EC50) was 10 units/ml IFN-γ. The inhibition of cell proliferation by IFN-γ was an irreversible process since removal of IFN-γ from the medium 3 h after addition failed to restore proliferation. This was confirmed by the observed reduction in the colony forming efficiency of NHEK cells from 43% to ≤1% after IFN-γ treatment (Table I). These results indicate that IFN-γ commits NHEK cells to a state of irreversible growth arrest. The growth inhibitory action was specific for IFN-γ since the addition of 1000 units/ml IFN-α had little effect on the proliferation of NHEK cells (Fig. 1A). The inhibition of the growth of NHEK cells by IFN-γ was accompanied by changes in the expression of several growth control genes. As shown in Fig. 1B, treatment of

NHEK cells with IFN-γ caused a ≥95% reduction in the level of p53, cdc2, and E2F-1 mRNA. In contrast, the level of Rb mRNA was not affected at all whereas IFN-γ caused a reduction of about 50% in the level of c-myc mRNA. The responsiveness of c-myc expression to IFN-γ varied with no change being observed in some experiments.

NHEK cells treated with IFN-γ acquired a squamous phenotype and became more adhesive to the substratum (not shown). These observations are consistent with an induction of squamous cell differentiation in IFN-γ-treated NHEK cells. To investigate this further, we tested the effect of IFN-γ on the expression of two established squamous cell-specific genes, transglutaminase type I and cornifin (13, 18, 35). Addition of
IFN-γ to cultured NHEK cells caused a dose-dependent increase in type I (particulate) transglutaminase activity and cornifin protein (Fig. 2, A and B). The EC_{50} for the induction of transglutaminase activity and cornifin was estimated to be 25 and 40 units/ml of IFN-γ, respectively. The increase in transglutaminase was time-dependent (Fig. 2C). After 3 days of treatment, IFN-γ caused a 10–14-fold increase in transglutaminase type I activity above control levels. Previously it was shown that increasing Ca^{2+} concentration in the medium (1.8 instead of 0.15 mM) enhanced transglutaminase type I expression in confluent, differentiating cultures of NHEK cells whereas a high Ca^{2+} concentration had little effect in undifferentiated (exponential phase) cultures (34). These findings suggested that Ca^{2+} by itself does not trigger differentiation in NHEK cells but can enhance the expression of squamous cell-specific genes in cells already committed to differentiate (37). The observed effects of Ca^{2+} on IFN-γ-treated and untreated cell s are in agreement with this interpretation; high Ca^{2+} concentration in the medium increased the IFN-γ-induced transglutaminase activity (Fig. 2, A and C) whereas high calcium by itself caused only a slight increase. Since cultures treated with high Ca^{2+} continue to grow and are at a higher cell density (but still at subconfluence) than IFN-γ-treated cultures at the time cells are harvested, the small increase in squamous-specific genes by Ca^{2+} may be due to increased expression in a small fraction of cells already committed to squamous differentiation. The IFN-γ-induced changes in transglutaminase type I activity and cornifin protein were shown to be related to an increase in the level of corresponding mRNA (Fig. 3). The presence of high Ca^{2+} concentration slightly increased the level of transglutaminase type I and cornifin mRNA whereas simultaneous treatment of NHEK cells with IFN-γ and Ca^{2+} had a synergistic effect on transglutaminase type I and cornifin mRNA levels.

Previous reports indicated that the controls of growth and differentiation are interrelated (2, 5). To study the link between growth arrest and the induction of the squamous-differentiated phenotype further, we examined the action of IFN-γ on several growth regulatory and squamous cell-specific genes as a function of the duration of IFN-treatment. As shown in Fig. 4A treatment of NHEK cells with IFN-γ caused a rapid decline in the level of cdc2 and E2F-1 mRNAs. This decrease coincided with the inhibition of the incorporation of [3H]-thymidine (Fig. 4A). In contrast, the changes in the level of transglutaminase type I and cornifin mRNA were observed after a lag time of more than 12 h (Fig. 4B). These findings indicate that changes in the expression of growth regulatory genes occur more rapidly than that of squamous cell-specific genes and suggest that growth arrest precedes the induction of differentiation-specific genes. These observations are consistent with the hypothesis that in normal cells the control of growth arrest and differentiation are closely linked (5).

### Table 1: Effect of IFN-γ, retinoic acid, and TGF-β1 on the colony forming efficiency in NHEK cells

| Treatment | CFE (×10^-3) |
|-----------|-------------|
| NA        | 43 (±4)     |
| IFN-γ     | ≤0.1        |
| RA        | 39 (±5)     |
| TGF-β1    | 34 (±5)     |
| IFN-γ + RA | ≤0.1       |
| IFN-γ + TGF-β1 | ≤0.1 |

Several studies have reported that retinoic acid inhibits squamous differentiation in phorbol ester-treated and confluent cultures of NHEK cells (5, 38). It has been demonstrated that retinoids do not block irreversible growth arrest but abol-
were treated with 1.8 units/ml of glutaminase type I and examined by Northern blot analysis using 32P-labeled probes for transglutaminase type I (TGase I), cornifin, and GPDH.

The addition of retinoic acid to IFN-γ treated NHEK cells demonstrated an antagonistic action between these two cytokines. Although TGF-β1 did not prevent the IFN-γ-induced irreversible growth arrest as determined by colony forming efficiency (Table I), it suppressed the induction of the squamous-specific genes, cornifin (Fig. 6) and transglutaminase type I (Fig. 7). Additionally, while suppressing the IFN-γ-induced transglutaminase type I activity increasing concentrations of TGF-β1 enhanced type II transglutaminase activity (Fig. 7). The latter is consistent with previous observations (20) showing induction of transglutaminase type II by TGF-β1 in NHEK cells and indicates that the action of TGF-β1 on the expression of transglutaminases prevails over that of IFN-γ.

The antagonism between IFN-γ and retinoic acid-TGF-β1 on differentiation was reflected in changes in the mRNA levels of the differentiation-specific genes (Fig. 8). The increase in transglutaminase type I and cornifin mRNA by IFN-γ was abrogated by both retinoic acid and TGF-β1. The action of TGF-β1 on gene expression was dominant over the effect of IFN-γ as demonstrated by the effects on transglutaminase type II and collagen IV. TGF-β1 either in the presence or absence of IFN-γ increased the level of type II transglutaminase mRNA. Similarly, the expression of collagen α1(IV), another gene induced by TGF-β1 in NHEK cells (20, 42), was also enhanced by TGF-β1 in the presence of IFN-γ.

The level of cdc2 mRNA in NHEK cells treated under various conditions correlated closely with the extent of [3H]thymidine incorporation (Fig. 8). The down-regulation of cdc2 mRNA by IFN-γ was not reversed by retinoic acid and supports the conclusion that retinoic acid does not prevent growth arrest (Table I). Although both IFN-γ and TGF-β1 caused growth ar-

Fig. 3. Effect of IFN-γ and calcium ions on transglutaminase type I and cornifin mRNA levels. NHEK cells (2 x 10⁶ cells/60-mm dish) were treated with 1000 units/ml IFN-γ in the presence or absence of 1.8 units/ml CaCl₂. After 3 days of incubation, total RNA was isolated and examined by Northern blot analysis using 32P-labeled probes for transglutaminase type I (TGase I), cornifin, and GPDH.

Fig. 4. Comparison of the effects of IFN-γ on the level of several growth regulatory and squamous cell-specific mRNAs as a function of the duration of IFN-γ treatment. NHEK cells were treated with 1000 units/ml IFN-γ and at different time intervals after the addition of IFN-γ total RNA was isolated and used for Northern blot analysis. The relative mRNA level was determined by densitometry of Northern blots probed for cdc2 (A) and E2F-1 and transglutaminase type I (TGase I) (B) and cornifin. Densitometric values were corrected for possible differences in total RNA loading using values obtained from probing with glyceraldehyde-3-phosphate dehydrogenase. The incorporation of [3H]thymidine into DNA was also determined (A).

Fig. 5. Inhibition of IFN-γ-induced transglutaminase type I expression by retinoic acid. NHEK cells were treated with IFN-γ (1000 units/ml) in the presence of retinoic acid at the concentrations indicated. Three days later, cells were harvested and assayed for particulate (type I) transglutaminase activity (solid line), vehicle-treated NHEK cells (broken line), and cornifin. Densitometric values were corrected for possible differences in total RNA loading using values obtained from probing with glyceraldehyde-3-phosphate dehydrogenase. The incorporation of [3H]thymidine into DNA was also determined (A).

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rest in NHEK cells, only the growth arrest by TGF-β1 was accompanied by a ≥95% reduction in c-myc mRNA levels (not shown) whereas cdc2 mRNA was down-regulated by both agents. When NHEK cells were treated with IFN-γ and TGF-β1 simultaneously the action of TGF-β1 on c-myc expression prevailed (not shown). These data support the hypothesis that retinoic acid and TGF-β1 cooperate to suppress IFN-γ-induced expression of squamous-specific genes but do not prevent IFN-γ-mediated irreversible growth arrest.

Squamous carcinoma cells exhibit defects in the mechanisms that control growth and differentiation. To determine whether squamous carcinoma cells and transformed epidermal cells are altered in their response to IFN-γ, we examined the effect of IFN-γ on the incorporation of [3H]thymidine and compared this with the effects on cdc2 and transglutaminase type I mRNA expression. IFN-γ had little effect on the incorporation of [3H]thymidine and on the level of cdc2 mRNA in the carcinoma cell lines K14, SCC13, and SQCC/Y1 (Fig. 9). In addition, IFN-γ did not induce expression of the squamous cell-specific gene, transglutaminase type I. These results suggest that these cells are resistant to IFN-γ-induced squamous differentiation. This resistance appears not to be due to the inability to express IFN-γ receptors since mRNA for these receptors was detected in all these cells (Fig. 9).

DISCUSSION

In this study, we show that treatment of NHEK cells with IFN-γ induces two stages characteristic of squamous differentiation, irreversible growth arrest and expression of squamous cell-specific markers. These findings indicate that IFN-γ is a potent inducer of squamous cell differentiation and are consistent with the ultrastructural changes reported previously in

FIG. 6. Inhibition of IFN-γ-induced cornifin expression by retinoic acid and TGF-β1. NHEK cells were treated with IFN-γ (IFNγ, 500 units/ml) in the absence and presence of either retinoic acid (RA, 100 nm) or TGF-β1 (100 pg) for 3 days before cells were collected and proteins analyzed by immunoblotting using the anti-cornifin antiserum SQ37B-Ab. NA, vehicle-treated NHEK cells.

FIG. 7. Antagonism between IFN-γ and TGF-β1. NHEK cells were treated with IFN-γ (500 units/ml) in the presence of TGF-β1 at the concentrations indicated. After 3 days of incubation, cells were harvested and assayed for type I (particulate, hatched bars) and type II (soluble, solid bars) transglutaminase activity.

FIG. 8. Comparison of the effects of IFN-γ, TGF-β1, and retinoic acid on the expression of growth regulatory and squamous cell-specific genes in NHEK cells. Cells were treated in the presence of IFN-γ (150 units/ml), retinoic acid (1 µM), or TGF-β1 (100 pg) for 3 days before cells were collected and RNA was isolated. Total RNA was then examined by Northern blot analysis using 32P-labeled probes for transglutaminase type I and II (TGate), cornifin, collagen IV (Coll. IV) and cdc2. GPDH was used as a control. In separate dishes the incorporation of [3H]thymidine into DNA was determined and is presented as mean ± S.E.

IFN-γ-treated NHEK cells (33). The irreversible growth arrest of NHEK cells induced by IFN-γ was accompanied by a decrease in the levels of cdc2, p53, and E2F-1 mRNA whereas the levels of c-myc and Rb mRNA were not greatly altered. The decrease in the expression of cdc2 and E2F-1 mRNAs appears to precede the increase in the expression of two differentiation-specific genes, transglutaminase type I and cornifin. Our results are consistent with the hypothesis that the control of growth arrest and expression of squamous cell-specific genes are separate but interdependent processes. A similar coupling has been demonstrated previously for squamous differentiation in phorbol ester-treated NHEK cells and in confluent NHEK cultures (5, 22). In several other cell systems that undergo terminal differentiation, a link between proliferation and differentiation has been established (6). In muscle cell and adipocyte differentiation, specific “master genes,” like myoD and CEBP, respectively, have been shown to induce growth arrest as well as the expression of certain differentiation-specific genes and appear to be involved in coupling growth arrest and expression of the differentiated phenotype (43, 44). Although the existence of such putative master genes in squamous differentiation has been postulated, such genes have yet to be identified (5).

The induction of transglutaminase type I and cornifin was delayed compared to IFN-γ-induced growth arrest. Therefore, the induction of squamous cell-specific genes by IFN-γ may be rather distal from the initial activation of its receptor and may
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![Graph showing comparison of IFN-\(\gamma\) effects on NHEK and several squamous cell carcinoma cell lines.](image)

not involve \(\gamma\)-activated site elements in the promoter of these genes. Instead, the IFN-\(\gamma\)-induced increase in squamous-specific genes may be a consequence of an alteration in the rate of transcription of genes (possibly the master genes mentioned above) that initiate events at an earlier stage in the differentiation process. The fact that no \(\gamma\)-activated site elements are present in a 3-kb promoter region of the transglutaminase type I gene (7) could support this hypothesis. Alternatively, the action of IFN-\(\gamma\) in NHEK cells may involve other signaling pathways such as the activation of protein kinase C (31) or increased turnover of sphingomyelin (32). Since phorbol ester-mediated activation of protein kinase C has been shown to induce squamous differentiation in NHEK cells (5, 22), the induction of squamous differentiation by IFN-\(\gamma\) could be mediated by the activation of protein kinase C. However, such a mechanism is not consistent with the observation that bryostatin, which blocks phorbol ester-induced squamous differentiation, has been shown to be resistant to phorbol ester-induced squamous differentiation (22). In this study, we show that several squamous cell carcinoma cell lines are not growth arrested and do not exhibit a reduced level of cdc2 mRNA after IFN-\(\gamma\) treatment. In addition, no increase in the level of transglutaminase type I mRNA was observed. These results indicate that IFN-\(\gamma\) does not induce squamous differentiation in these cells. This inability to induce differentiation is not due to a lack of IFN-\(\gamma\) receptor expression since IFN-\(\gamma\) receptor mRNA was detected in the carcinoma cells. In addition, these cell lines do not have an intrinsic defect in the IFN-\(\gamma\) signaling pathway since the induction of guanylate-binding protein by IFN-\(\gamma\) was not impaired in these cells. Since these cells also do not undergo differentiation at confluence or when treated with phorbol esters (22), the defect in the control of squamous differentiation may be at a point that is common to the different signaling pathways.

Retinoic acid was unable to prevent the IFN-\(\gamma\)-induced irreversible growth arrest but effectively antagonized the induction of the squamous cell-specific genes, transglutaminase type I and ccdc2. Similarly, retinoic acid has been shown previously to inhibit the induction of squamous cell-specific genes in phorbol ester-treated NHEK cells and in confluent cultures without preventing irreversible growth arrest (5, 20). These results suggest that retinoic acid affects specific stages during squamous differentiation. Retinoic acid may inhibit the induction of differentiation by phorbol esters and IFN-\(\gamma\) at a step that is common to both signal transduction pathways. Recently, we reported that phorbol esters and retinoic acid regulate transglutaminase type I expression at the transcriptional level (7).

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