Expression of Interferon-β is Associated with Growth Arrest of Murine and Human Epidermal Cells

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The cytokine interferon-β is a regulator of cell replication and function, including invasion and induction of angiogenesis. The goal of this study was to determine whether the expression of interferon-β by cells in the epidermis correlated with terminal differentiation. *In situ* hybridization analysis and immunohistochemical staining of formalin-fixed, paraffin-embedded specimens of normal human and murine epidermis and human and murine skin tumors of epithelial origin revealed that only differentiated, nondividing cells of the epidermis expressed interferon-β protein. Keratinocyte cultures established from the epidermis of 3 d old mice were maintained under conditions permitting continuous cell division or induction of differentiation. Continuously dividing cells did not produce interferon-β whereas nondividing differentiated cells expressing keratin 1 did. Growth-arrested, undifferentiated keratinocytes also expressed interferon-β protein. Neutralizing interferon-β in the culture medium inhibited differentiation, but the addition of exogenous interferon-β did not stimulate differentiation. These data indicate that interferon-β is produced by growth-arrested, terminally differentiated keratinocytes. *Keywords*: *angiogenesis/differentiation. J Invest Dermatol 112:802–809, 1999*

The interferons (IFN) are a heterogeneous family of secreted proteins with the capacity to exert pleiotropic effects on cell functions and render cells resistant to infection by microorganisms (Isaacs and Lindenmann, 1957; De Maeyer-Guignard and De Maeyer, 1985; Tamm *et al*, 1987; Gresser, 1990; Kalvakolanu and Borden, 1996). Type I IFN-α and IFN-β, formerly classified as leukocyte and fibroblast IFN, respectively, bind to the same cell surface receptor (Branca and Baglioni, 1981) IFN-α and IFN-β induce a similar pattern of cellular responses, yet certain reactions can be stimulated only by IFN-β, presumably by the phosphorylation of a receptor-associated protein that is uniquely responsive to IFN-β (Abramovich *et al*, 1994; Uze *et al*, 1995). In response to viral infection, epithelial cells and fibroblasts secrete IFN-β into the organ microenvironment, where it can reach high concentrations (Dianzani, 1992). Although IFN are thought of as inducible proteins, their presence has been detected in different human tissues in the absence of any obvious inducer (Tovey, 1988). IFN-β was discovered several decades ago based on its anti-viral activity (Isaacs and Lindenmann, 1957). Subsequent investigations, however, demonstrated that IFN-β functions as an autocrine growth inhibitor during hematopoietic cell differentiation by binding to cell surface receptors and switching on the expression of members of the IFN-inducible gene family (Resnitzky *et al*, 1986). Treatment with exogenous IFN-β or IFN-α has been shown to produce cytostasis and promote terminal differentiation of epidermal cells (Nickoloff *et al*, 1984; Yaar *et al*, 1985; Stadler *et al*, 1986). IFN-β production has been associated with differentiation in several cell systems including pluripotent P19 embryonal carcinoma cells (Belhumeur *et al*, 1993), human U937 leukemia cells (Yarden *et al*, 1984), and Friend erythroleukemia cells (Friedman-Einat *et al*, 1982).

Recently, we reported that differentiated epithelial cells lining organs exposed to environmental stimuli constitutively express IFN-β (Bielenberg *et al*, 1998a). We have also found that expression of IFN-β protein is inversely correlated with angiogenesis in cutaneous infantile hemangiomas and that the eventual involution of these extremely angiogenic tumors coincides with increased expression of IFN-β in the epidermis overlying these lesions (Bielenberg *et al*, 1999). Hyperplasia and cutaneous angiogenesis induced by ultraviolet (UV) irradiation also correlate with a loss of IFN-β protein expression in the epidermis of mice (Bielenberg *et al*, 1998b). Because IFN-β can regulate such diverse homeostatic processes as cellular proliferation (Clemens and McNurlan, 1985; Heyns *et al*, 1985), differentiation (Clemens and McNurlan, 1985; Rossi, 1985), immunity (Lengyel, 1982; De Maeyer-Guignard and De Maeyer, 1985; Gresser *et al*, 1991), and angiogenesis (Sidky and Borden, 1987; Dvorak and Gresser, 1989; Singh *et al*, 1995), we wished to determine its constitutive expression in epithelial cells and whether its production was correlated with their differentiation. We therefore examined the expression of IFN-β in normal human and murine epidermis as well as in human and murine skin tumors of epithelial origin. To test our hypothesis that expression of IFN-β is associated with terminal differentiation, we used primary cultures of epidermal cells whose differentiation can be induced by changing

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Abbreviations: ara-C, cytosine-B-D-arabinofuranoside; PCNA, proliferating cell nuclear antigen.

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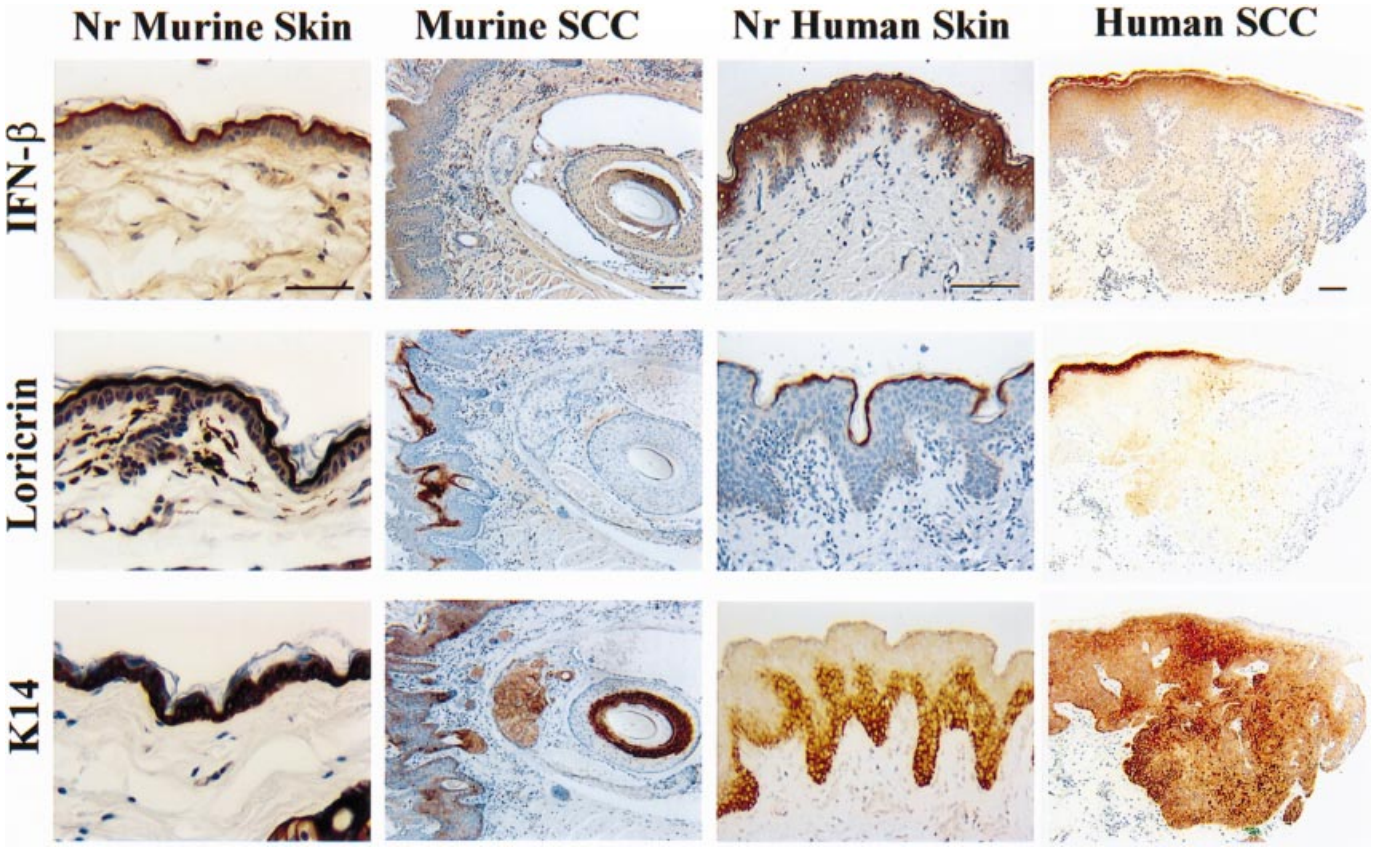

Figure 1. Localization of IFN-β protein and differentiation-associated antigens in normal epidermis and squamous cell carcinomas (SCC). In normal murine or human epidermis, IFN-β protein is expressed in the suprabasal layers. Loricrin is expressed in the outer granular and cornified layers. K14 is expressed in all epidermal layers in the skin of mice and in basal cells in the human skin. Dividing murine (well differentiated) and human SCC cells all express K14 but have decreased expression of loricrin and IFN-β protein. *Scale bars*: 50 μM.

the calcium concentration in the culture medium (Hennings *et al*, 1980; Yuspa *et al*, 1989). The data show that only keratinocytes that undergo terminal differentiation, identified by the expression of differentiation-associated antigens (K1 and loricrin) and a cornified envelope (Roop *et al*, 1987; Yuspa *et al*, 1989) or keratinocytes whose growth is arrested by cytostatic agents, express IFN-β.

MATERIALS AND METHODS

Keratinocyte cultures Primary murine keratinocytes isolated from 3 d old BALB/C mice (Hennings *et al*, 1980) were plated at a density of 5×10^6 cells per 60 mm dish (Corning Glass Works, Corning, NY) or 3×10^6 cells per ProbeOn slide (Fisher Scientific, Houston, TX) and maintained in Eagle's minimum essential medium (Bio-Whittaker, Walkersville, MD) containing 8% chelated fetal bovine serum (Intergen, Purchase, NY), 0.05 mM Ca^{2+}, and 20 U penicillin–streptomycin (Gibco, Grand Island, NY) per ml. Differentiation of the keratinocytes was induced by incubation in medium containing 0.12 mM Ca^{2+} (Roop *et al*, 1987). To inhibit proliferation, the keratinocyte cultures were treated with medium containing 1 ng TGF-β (R&D Systems, Minneapolis) per ml or 100 μM cytosine-B-D-arabinofuranoside (ara-C; Sigma, St. Louis, MO) (Weinberg *et al*, 1995). To identify dividing cells, the cultures were treated for 18 h with 50 μM BrdU (Sigma). In other experiments, keratinocytes grown in medium containing 0.05 mM Ca^{2+} or 0.12 mM Ca^{2+} were treated with 10^2–10^4 U recombinant murine IFN-β (Access Biomedical, San Diego, CA) per ml. To neutralize the effect of IFN on differentiation, keratinocytes were incubated with 10–100 neutralizing units per ml murine or human IFN-β antibody 1 h prior to and during elevation of the calcium concentration from 0.05 mM to 0.12 mM.

Tissue specimens Ten formalin-fixed, paraffin-embedded archival surgical specimens of human squamous cell carcinomas and normal skin from patients treated at The University of Texas M.D. Anderson Cancer Center were chosen at random. Samples of skin were collected from transgenic mice expressing the human papillomavirus type 16 early region genes under the control of the human K-14 promoter (K14-HPV16) back-crossed into the FVB/n genetic background. The epidermis of these mice undergoes progression from normal epidermis to hyperplasia, high-grade dysplasia and, finally, invasive squamous carcinoma (Arbeit *et al*, 1994; Coussens *et al*, 1996). Skin samples from K14-HPV16 mice at various stages of disease progression were fixed in 3.75% paraformaldehyde and embedded in paraffin at the University of California, San Francisco, CA.

Antibodies Rabbit anti-mouse K1, K14, and loricrin antibodies as well as guinea-pig anti-mouse K1 antibody were the generous gift of Dr. Dennis Roop (Baylor College of Medicine, Houston, TX). These antibodies are now available from Berkeley Antibody (Richmond, CA). Affinity-purified polyclonal rabbit anti-mouse IFN-β and monospecific polyclonal rabbit anti-human IFN-β antibodies were purchased from Access Biomedical; monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody, clone PC10, was purchased from Dako (Carpinteria, CA); monoclonal mouse anti-BrdU antibody (IgG1), clone B44, was purchased from Becton Dickinson (San Jose, CA); rabbit anti-actin antibody was purchased from Sigma; peroxidase-conjugated $F(ab')_2$ goat anti-rabbit IgG $F(ab')_2$, Texas Red-conjugated $F(ab')_2$ goat anti-rabbit IgG $F(ab')_2$, and aminomethyl Coumarin acetate-conjugated $F(ab')_2$ goat anti-guinea-pig IgG $F(ab')_2$ were purchased from Jackson Research Laboratories (West Grove, PA); peroxidase-conjugated rat anti-mouse IgG2a was purchased from Serotec, Harlan Bioproducts for Science (Indianapolis, IN); and peroxidase-conjugated rat anti-mouse IgG1 was purchased from PharMingen (San Diego, CA).

Immunocytochemistry Acetone-fixed, phosphate-buffered saline (PBS)-washed, tissue culture slides were placed in a humidified chamber and incubated with a solution of 3% H_2O_2 in methanol solution for 12 min at room temperature to block endogenous peroxidases. The slides were washed three times with PBS (pH 7.5), blocked for 20 min at room temperature in PBS supplemented with 1% normal goat serum and 5% normal horse serum (protein blocking solution), and incubated with primary antibody overnight at 4°C. The slides were then rinsed three times with PBS, incubated for 10 min in protein blocking solution, and incubated for 1 h at room temperature in the corresponding peroxidase-conjugated secondary antibody. Next, the slides were washed and incubated with

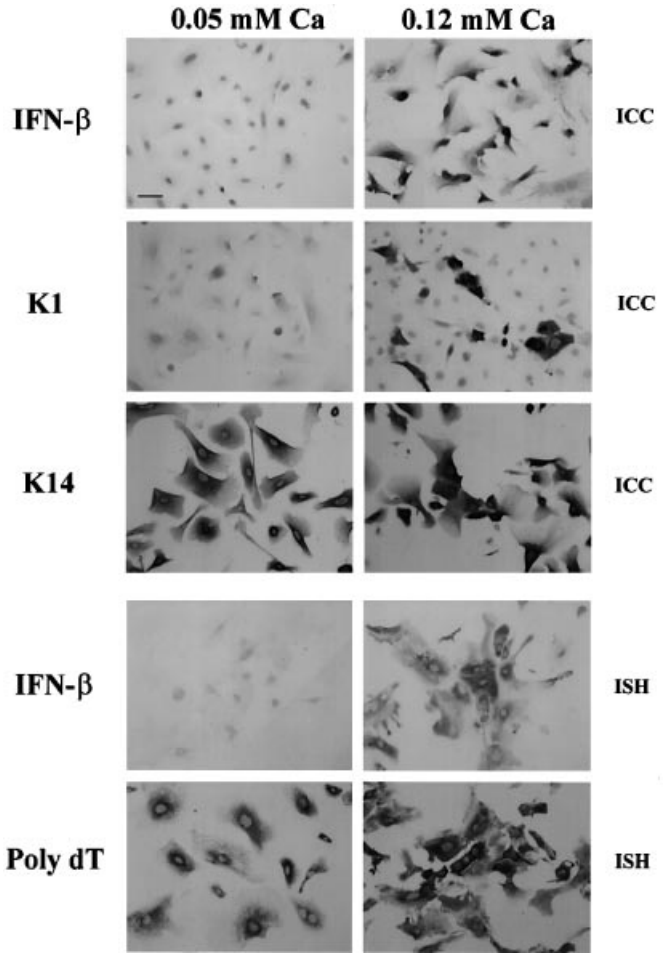

Figure 2. Expression of IFN-β mRNA and protein in differentiated keratinocytes. Primary cultures of keratinocytes were grown in medium containing 0.05 mM Ca^{2+} (undifferentiated) or 0.12 mM Ca^{2+} (differentiated). By immunocytochemistry (ICC), both undifferentiated and differentiated keratinocytes expressed K14 protein, but only differentiated keratinocytes expressed IFN-β and K1 proteins. ISH analysis revealed higher levels of IFN-β mRNA in differentiated keratinocytes than in undifferentiated keratinocytes. All keratinocytes had intact mRNA as shown by poly $d(T)_{20}$ staining. *Scale bars*: 50 μM.

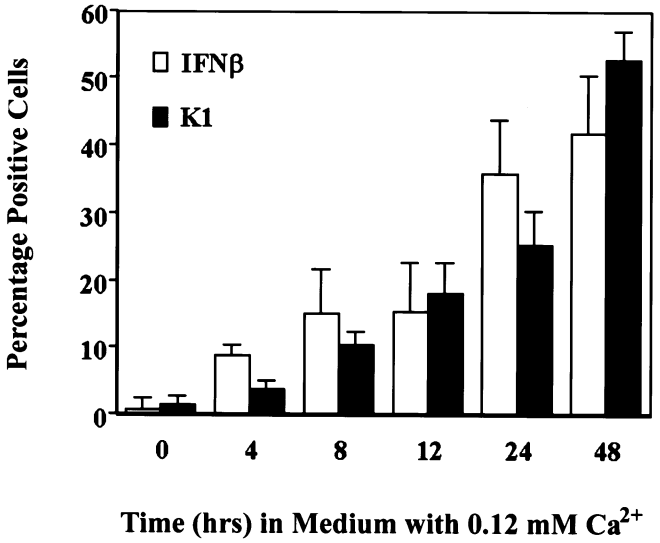

Figure 3. Kinetics of keratinocyte differentiation and expression of IFN-β protein. Primary keratinocytes grown on glass slides were incubated in medium containing 0.12 mM Ca^{2+} for 4–48 h. The slides were fixed and stained with antibodies against IFN-β or K1. The average percentage of cells expressing IFN-β or K1 proteins at each time point was determined by counting 15 random 100× fields. Control cells were grown in 0.05 mM Ca^{2+}-containing medium.

stable diaminobenzidine (DAB; Research Genetics, Huntsville, AL). Staining (brown precipitate) was monitored under a bright field microscope, and washing with distilled water stopped the reaction. For single antibody labeling experiments, the slides were counterstained with Gill's 3 hematoxylin (Sigma) and mounted with Universal Mount (Research Genetics). For double antibody labeling experiments to detect BrdU, the slides were washed with PBS after DAB, treated with 1% Triton X-100 in PBS for 8 min, and rinsed again with PBS. The slides were then incubated with 2 M HCl in PBS at 37°C for 30 min and rinsed with PBS. Next, samples were treated with protein blocking solution for 10 min at room temperature and incubated overnight at 4°C with primary antibody (anti-BrdU). The slides were then washed with PBS, blocked with protein blocking solution, and incubated with peroxidase-conjugated secondary antibody (rat anti-mouse IgG1) for 1 h at room temperature. The slides were finally incubated in a second chromogen, 3-amino-9-ethylcarbazole (BioGenex, San Ramon, CA) for 5–10 min, washed, counterstained, and mounted as described above. A positive reaction is seen as red nuclear staining. The concentration of primary and secondary antibodies was determined previously and single antibody labeling of samples was included as a control. Samples exposed to secondary antibody alone showed no specific staining.

Immunohistochemistry Paraffin-embedded tissues were cut into 5 μm sections, mounted on positively charged Superfrost slides (Fisher Scientific), and allowed to dry overnight at room temperature. Sections were deparaffinized in xylene followed by a graded series of alcohols and rehydrated in PBS (pH 7.5). Sections were then incubated with pepsin (Biomeda, Foster City, CA) for 30 min at 37°C and treated as above for immunocytochemical analysis. DAB chromogen was used in single antibody labeling experiments. Samples used for double antibody labeling experiments were microwaved 5 min for citrate buffer "antigen retrieval" (Shi *et al*, 1991) in place of pepsin treatment. The samples were incubated with the second primary antibody followed by peroxidase-conjugated secondary antibody (anti-PCNA; rat anti-mouse IgG2a) and treated with True Blue chromogenic substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Slides were briefly washed with distilled water and mounted with Permount (Fisher Scientific). A positive reaction is seen as blue nuclear staining.

Immunofluorescence Acetone-fixed, PBS-washed, tissue culture slides were treated as described above for immunohistochemical analysis except that on the second day, the slides were incubated for 1 h at room temperature with a Texas Red-conjugated secondary antibody and kept in a light-protected chamber. The slides were then washed with PBS, blocked with protein blocking solution, and incubated overnight at 4°C with another primary antibody. On the third day, the slides were washed with PBS, blocked with protein blocking solution, and incubated for 1 h at room temperature with an aminomethyl Coumarin acetate-conjugated secondary antibody. The slides were washed with PBS and mounted using a 9:1 glycerol/PBS solution containing 2.1% propyl gallate. The reactions were confirmed by fluorescent microscopy. Control slides exposed to one or both secondary antibodies did not show fluorescent staining.

Oligonucleotide probes Two oligonucleotide probes were designed to be complementary to the IFN-mRNA transcript. The sequences of the 21-mer probes for IFN-were 5′CGT-CCT-TTC-TTG-GAG-CTG-GAG-3′ and 5′CAC-TGT-CTG-CTG-GTG-GAG-TTC-3′ (both 57.1% GC content). The specificity of the oligonucleotide sequences was first determined by a GenEMBL database search using the Genetics Computer Group sequence analysis program (GCG, Madison, WI), based on the FastA algorithm; the sequences showed 100% homology with the target IFN-β gene and minimal homology with nonspecific mammalian gene sequences (Pearson and Lipman, 1988). A poly $d(T)_{20}$ oligonucleotide was used to verify the integrity of the mRNA in each sample (Bucana *et al*, 1993; Radinsky *et al*, 1993). All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling using standard phosphoramidite chemistry [TAG-BBB-(TGA)-BBB] (Research Genetics) (Caruthers *et al*, 1982). The lyophilized probes were reconstituted to a 1 μg per μl stock solution in 10 mM Tris (pH 7.6) and 1 mM EDTA. Each probe was titrated using a known positive tissue or cell line to determine the optimal working dilution. The two IFN-β probes were used together, each at a working dilution of 1:200 in Probe Diluent (Research Genetics); and a dilution of 1:1000 was used for the poly $d(T)_{20}$ probe.

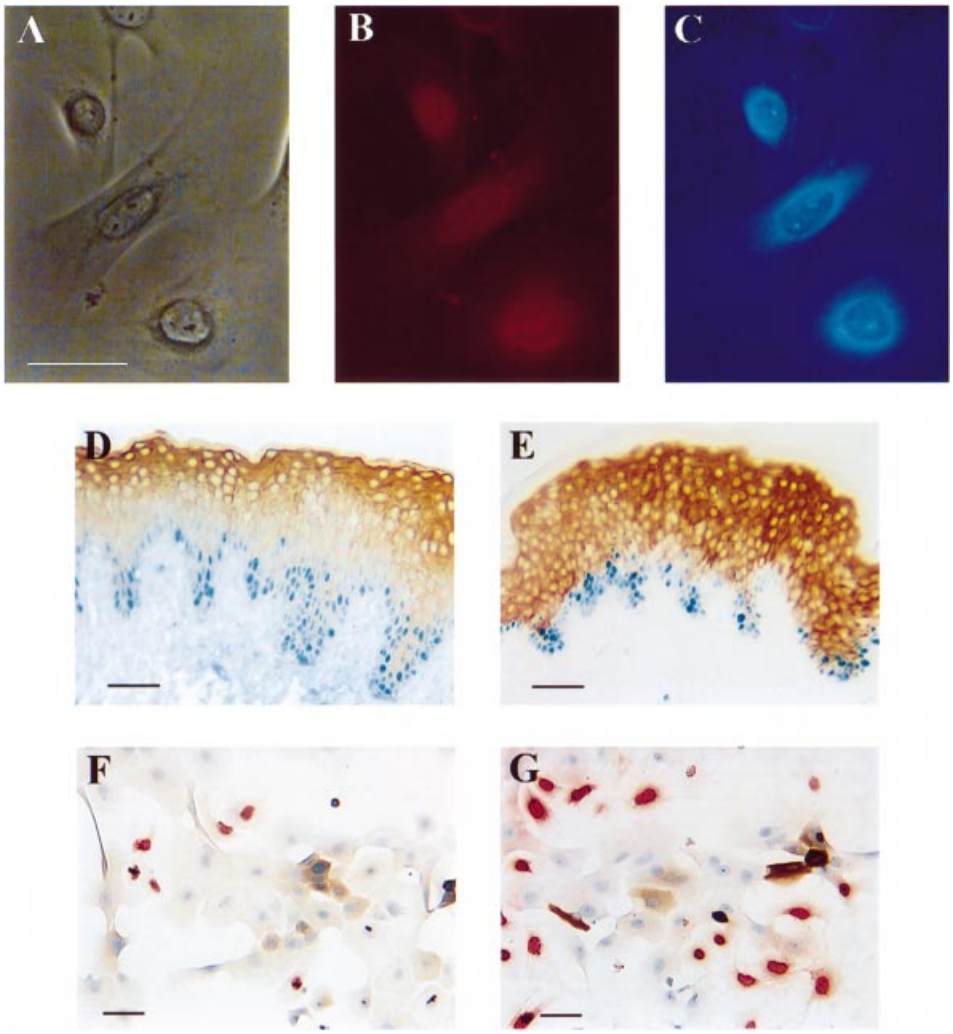

Figure 4. (*A–C*) Expression of IFN-β protein in nondividing, differentiated mouse keratinocytes. Double-labeling immunofluorescence analysis shows that differentiated keratinocytes coexpress IFN-β (Texas Red) and K1 (aminomethyl Coumarin acetate) proteins [(*A*) phase contrast; (*B*) IFN-β; (*C*) K1]. Double-labeling immunohistochemical analysis of human epidermis (*D*) shows that the nondividing, suprabasal epidermal cells express IFN-β protein (brown), whereas dividing, basal epidermal cells are PCNA-positive (blue). Keratinocytes in suprabasal layers (*E*) express the differentiation marker K1 (brown) but not PCNA. Primary mouse keratinocytes grown *in vitro* in 0.12 mM Ca^{2+}-containing medium and treated with BrdU also demonstrate that IFN-β (*F*) and K1 (*G*) proteins are not expressed by dividing cells (BrdU+, red stain). *Scale bars*: 50 μM.

***In situ* hybridization (ISH)** This was performed as described previously with minor modifications (Bucana *et al*, 1993; Radinsky *et al*, 1993). ISH was carried out according to the Microprobe manual staining system (Fisher Scientific, Pittsburgh, PA). Primary keratinocytes grown on sterile, silane-treated ProbeOn slides were fixed in 4% paraformaldehyde in RNase-free PBS for 20 min. The slides were placed in a Microprobe slide holder, washed twice with RNase-free Tris-buffered saline (TBS) [50 mM Tris–HCl, 150 mM NaCl (pH 7.6)], and incubated in 1% Triton X-100 for 5 min at room temperature. The slides were then washed three times with RNase-free TBS and treated with 0.2 M HCl for 3 min at 100°C. Hybridization of the biotinylated probe was carried out sequentially for 3 min at 100°C, 4 min at room temperature, and 45 min at 45°C. The samples were washed three times with 2 × standard saline citrate (0.3 M NaCl and 30 mM sodium citrate) for 2 min at 45°C and incubated with alkaline phosphatase-labeled avidin (Dako) for 30 min at 45°C. The slides were rinsed twice with TBS, treated with alkaline phosphatase enhancer (Biomeda) for 1 min, and finally incubated with the Fast Red chromogenic substrate (Research Genetics) for 30 min at 45°C. Slides were washed several times with distilled water and mounted with Universal Mount. ISH with the IFN-β and poly $d(T)_{20}$ probes was performed on each sample at the same time to eliminate reagent variability within samples. A positive reaction in this assay stains red. The control for endogenous alkaline phosphatase used chromogen in the absence of any oligonucleotide probes. To check the specificity of the hybridization signal, the following controls were used: (i) RNase pretreatment of cells; (ii) substitution of the anti-sense probe with a biotin-labeled sense probe; and (iii) competition assay with unlabeled anti-sense probes. A markedly decreased or absent signal was obtained after each of these procedures.

Western blotting Primary keratinocytes were washed with PBS containing 5 mM EDTA and 1 mM sodium-*o*-vanadate. Cells were scraped into lysis buffer [1% Triton X-100, 20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10% glycerol (vol/vol), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin and 0.15 U per ml aprotinin] and centrifuged to collect soluble protein. Proteins (20 μg per lane) were diluted with sample buffer [62.5 mM Tris–HCl (pH 6.8), 2.3% sodium dodecyl sulfate, 100 mM dithiothreitol, and 0.05% bromophenol blue], boiled, and separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were then transferred on to 0.45 μm nitrocellulose membranes. The filters were blocked with 3% bovine serum albumin in TBS [20 mM Tris–HCl (pH 7.5) and 150 mM NaCl], probed with antibodies against K1, K14, loricrin, or actin (1 μg per ml) diluted in TBS containing 0.1% Tween 20, incubated with horseradish peroxidase linked whole donkey anti-rabbit secondary antibody (Amersham, Arlington Heights, IL), and visualized by the enhanced chemiluminescence western blotting detection system (Amersham).

RESULTS

In the first set of experiments, we used immunohistochemistry to analyze the expression of IFN-β, loricrin, and keratin 14 (K14)

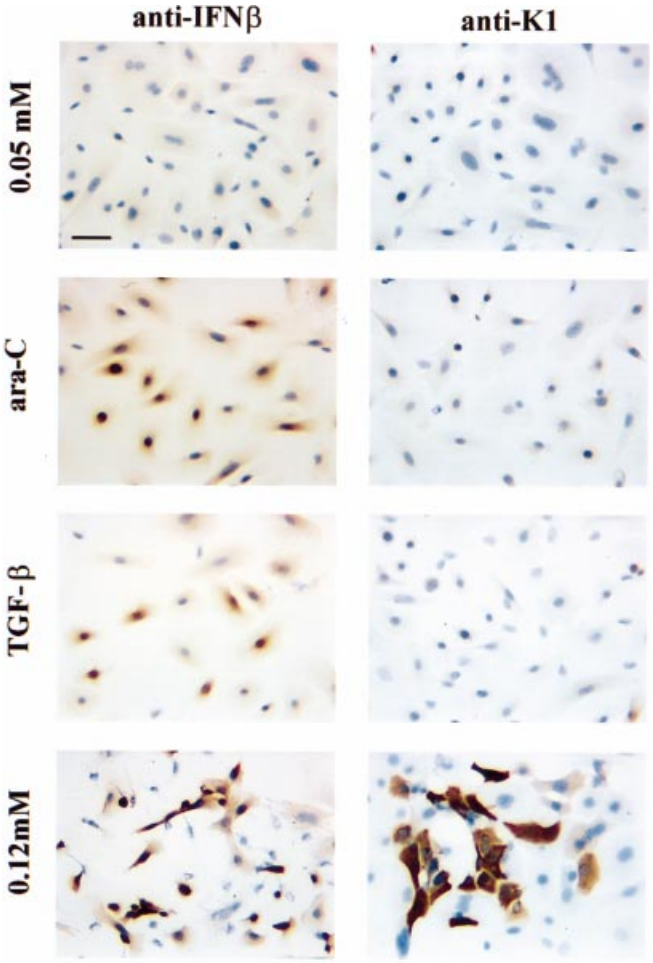

Figure 5. Expression of IFN-β protein in undifferentiated, growth-arrested mouse keratinocytes. Primary mouse keratinocytes grown *in vitro* in nondifferentiating medium (0.05 mM Ca^{2+}) do not express either IFN-β or K1 (immunocytochemistry). Keratinocytes whose growth was arrested with ara-C or TGF-β express IFN-β but not K1 proteins. Keratinocytes grown in differentiating medium (0.12 mM Ca^{2+}) used as a positive control express both IFN-β and K1 proteins.

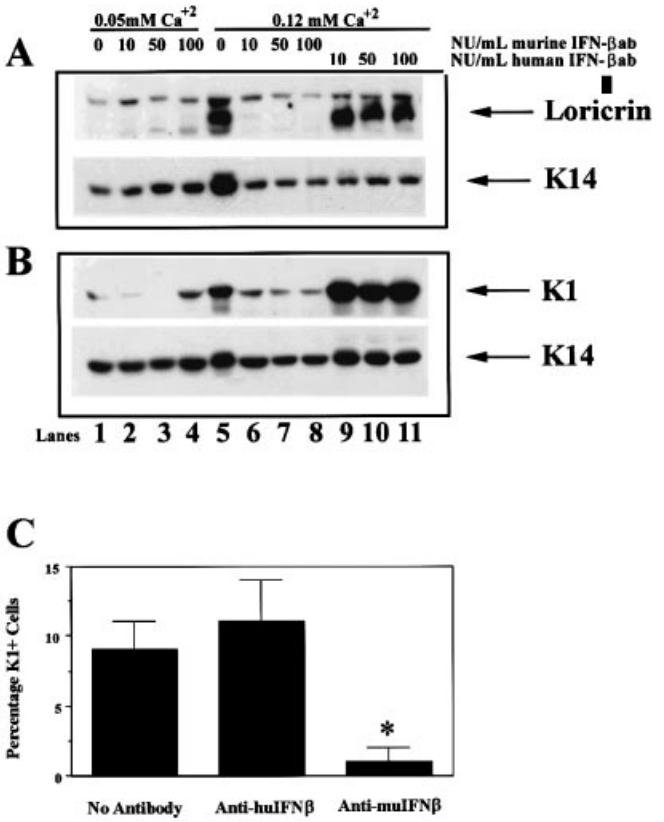

Figure 6. Neutralization of IFN-β protein inhibits differentiation of mouse keratinocytes. Western blot analysis demonstrates that keratinocytes grown in medium containing 0.05 mM Ca^{2+} and treated with 0 (*lane 1*), 10 (*lane 2*), 50 (*lane 3*), or 100 (*lane 4*) neutralizing units per ml of murine IFN-β antibody expressed little or no loricrin (*A*) or K1 (*B*) protein. In contrast, keratinocytes grown in medium containing 0.12 mM Ca^{2+} (*lane 5*) expressed both loricrin (*A*) and K1 (*B*). Keratinocytes incubated in medium containing 0.12 mM Ca^{2+} and treated with 10 (*lane 6*), 50 (*lane 7*), or 100 (*lane 8*) neutralizing units per ml murine IFN-β antibody did not differentiate, i.e., produce loricrin (*A*) or K1 (*B*) protein. Control keratinocytes grown in medium containing 0.12 mM Ca^{2+} media and treated with 10 (*lane 9*), 50 (*lane 10*), or 100 (*lane 11*) neutralizing units per ml human IFN-β antibody differentiated and produced loricrin (*A*) and K1 (*B*) proteins. K14, a 55 kDa protein, was expressed by all cells regardless of the culture condition and served as a loading control. (*C*) Primary keratinocytes were plated on glass slides, incubated in medium containing 0.12 mM Ca^{2+}, and treated with or without neutralizing antibodies against IFN-β. Immunocytochemical analysis was used to detect the number of K1-producing cells in each culture condition. Significantly fewer cells underwent differentiation when treated with antibodies to murine IFN-β. *$p < 0.0001$ by the Mann–Whitney nonparametric analysis of variance test in comparison with no antibody treatment.

protein in normal mouse and human skin and in epidermal squamous carcinomas from K14-HPV16 transgenic mice and human squamous cell carcinomas. In normal, quiescent tissues, negative regulators of cell proliferation and angiogenesis are usually dominant (Marks and Furstenberger, 1993; Fidler and Ellis, 1994; Iruela-Arispe and Dvorak, 1997). Indeed, the suprabasal layers of nontransgenic mice and human epidermis expressed high levels of IFN-β protein, whereas murine well-differentiated squamous cell carcinoma and human moderately differentiated squamous cell carcinoma did not (**Fig 1**). The normal epidermis expressed loricrin in the outer granular and cornified layers, whereas the squamous cell carcinomas did not. All specimens (normal and neoplastic epithelial cells) expressed K14 protein (**Fig 1**).

Expression of IFN-β protein in the suprabasal layers of the epidermis suggested that its expression was associated with cellular differentiation. To test this hypothesis, we established primary cultures of keratinocytes from the epidermis of 3 d old mice (Hennings *et al*, 1980). The cells were plated on glass slides and incubated for 2–3 d in medium containing 0.05 mM Ca^{2+}. The cultures were then washed and incubated for 24 h in medium containing 0.05 mM Ca^{2+} or 0.12 mM Ca^{2+}, fixed, and analyzed by immunocytochemistry and ISH for expression of IFN-β protein and mRNA and differentiation antigens. In medium containing 0.05 mM Ca^{2+}, the cells continued to proliferate, remained undifferentiated, and did not express IFN-β or keratin 1 (K1) proteins (**Fig 2**). In contrast, after 24 h of incubation in medium containing 0.12 mM Ca^{2+}, some of the cells began to differentiate, i.e., expressed K1 and IFN-β proteins (**Fig 2**). K14 protein was expressed by cells in either culture condition and was used as a positive control in all experiments (**Fig 2**). Cells incubated in medium with 0.05 mM Ca^{2+} did not express IFN-β mRNA, whereas some cells incubated with medium containing 0.12 mM Ca^{2+} did. Use of the poly $d(T)_{20}$ probe verified mRNA integrity.

To determine whether IFN-β protein was expressed by keratinocytes undergoing differentiation, we correlated the number of cells expressing K1 protein and cells expressing IFN-β. At time zero, murine keratinocytes were incubated in differentiating medium (0.12 mM Ca^{2+}). At various times thereafter, the cultures were fixed and stained for IFN-β or K1 proteins. The number of cells expressing either IFN-β or K1 increased with incubation time in differentiating medium (**Fig 3**). To determine whether the K1-positive cells also expressed IFN-β, we used immunofluorescence

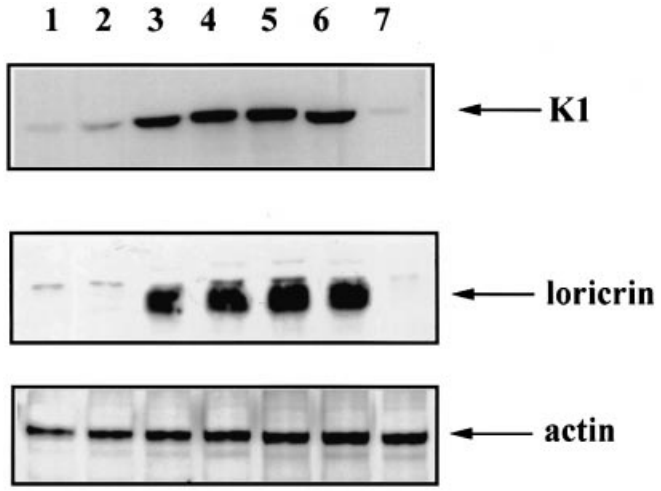

Figure 7. IFN-β treatment is insufficient for induction of differentiation in primary murine keratinocytes. Western blot analysis demonstrates that keratinocytes grown in medium containing 0.05 mM Ca^{2+} in the absence (*lane 1*) or presence of 1×10^4 U per ml exogenous murine IFN-β (*lane 2*) did not express K1 or loricrin. Cells grown in medium containing 0.12 mM Ca^{2+} (*lane 3*) expressed K1 and loricrin proteins, but the expression of these differentiation-specific markers was not elevated by treatment of cells for 24 h with 10^2 (*lane 4*), 10^3 (*lane 5*), or 10^4 (*lane 6*) U murine IFN-β per ml. Cells grown in medium with 0.05 mM Ca^{2+} and treated with 1 ng TGF-β per ml (*lane 7*) did not express K1 or loricrin. Actin, a ubiquitous 42 kDa protein expressed by all keratinocytes regardless of the culture conditions, served as a loading control.

analysis to detect both proteins. **Figure 4** illustrates that all cells expressing K1 also expressed IFN-β protein.

In the next set of studies, we determined whether cell division inversely correlated with expression of IFN-β. As terminally differentiated cells do not divide, we used immunohistochemistry and immunocytochemistry to identify the expression of IFN-β in dividing and nondividing keratinocytes under *in vivo* and *in vitro* conditions. Sections of normal human skin were labeled with antibodies against human IFN-β or antibodies against PCNA to identify cells in the S-phase of the cell cycle (Coltrera and Gown, 1991; Wolf and Dittrich, 1992; Iatropoulos and Williams, 1996). Proliferating cells were detected mainly in the basal layer of the epidermis or the layer immediately above the basal layer (**Fig 4**). The outer layers of the epidermis did not contain PCNA-positive cells. In contrast, expression of IFN-β protein was localized to cells in the suprabasal and granular layers (**Fig 4*D***). As expected, expression of K1 protein inversely correlated with cell division *in vivo* (**Fig 4*E***). To analyze further the coexpression of differentiation markers and IFN-β we cultured primary murine keratinocytes in medium containing 0.12 mM Ca^{2+}. Dividing cells were identified by treatment with 5-bromo-2′-deoxyuridine (BrdU) followed by staining with antibodies against BrdU (Coltrera and Gown, 1991; Wolf and Dittrich, 1992; Iatropoulos and Williams, 1996). Double-labeling revealed that keratinocytes expressing IFN-β (brown cytoplasm) did not stain with anti-BrdU antibodies (red nuclei) (**Fig 4*F***). Moreover, BrdU-positive cells (red nuclei) did not stain with antibodies against K1 (brown cytoplasm) (**Fig 4*G***). Thus, expression of IFN-β or K1 did not colocalize with BrdU expression, suggesting that only nondividing cells (presumably differentiated as well) expressed IFN-β. Single immunolabeling was performed to rule out steric hindrance or competition between antibodies. Reciprocal experiments were also performed to verify staining reactions.

To determine whether expression of IFN-β was associated with growth arrest or terminal differentiation of keratinocytes, we incubated primary cultures of murine keratinocytes in medium containing 0.05 mM Ca^{2+} (negative control) or medium containing 0.12 mM Ca^{2+} (positive control). To suppress cell division, the keratinocytes growing in medium with 0.05 mM Ca^{2+} were treated with transforming growth factor-β (TGF-β) or ara-C. Neither TGF-β nor ara-C induced differentiation of keratinocytes (**Fig 5**), in agreement with previously published reports (Weinberg *et al*, 1995). Growth-arrested keratinocytes did not express K1 but did express IFN-β protein (**Fig 5**).

To determine whether expression of IFN-β protein was necessary for differentiation of epidermal cells, we incubated primary keratinocytes in the presence or absence of IFN-β antibodies, isolated cytoplasmic proteins, and used western blot analysis to detect the differentiation-specific markers K1 and loricrin (**Fig 6**). Keratinocytes grown in medium containing 0.05 mM Ca^{2+} did not differentiate or produce IFN-β protein, and the addition of IFN-β antibodies had no effect on the expression of differentiated antigens (**Fig 6*A*, *B*,** *lanes 1–4*). About 10% of the keratinocytes grown in medium with 0.12 mM Ca^{2+} underwent differentiation as shown by the expression of loricrin (**Fig 6*A*,** *lane 5*) and K1 proteins (**Fig 6*B*,** *lane 5*). These differentiation antigens were absent or below detection level when IFN-β protein was neutralized by the addition of specific antibodies (**Fig 6**, *lanes 6–8*). Neutralizing antibodies specific for human IFN-β (which do not cross-react with murine IFN-β) did not inhibit differentiation of mouse keratinocytes (**Fig 6*A*, *B*,** *lanes 9–11*). The biologic activity of the anti-human IFN-β antibody was confirmed by its ability to neutralize the cytostasis of human A375 melanoma cells exposed to human IFN-β (data not shown). In **Fig 6**, parts (***A***) and (***B***) represent two separate experiments in which cells were treated independently with neutralizing antibodies. The loricrin blot (***A***) and K1 blot (***B***) were each stripped and reprobed with antibodies to K14, demonstrating protein expression and loading in each lane.

To estimate the number of cells undergoing differentiation within a population, we cultured primary murine keratinocytes on glass slides in medium with 0.12 mM Ca^{2+} and human or murine IFN-β antibodies. Using immunocytochemical techniques, the cultures were stained with antibodies against K1, and the average number of differentiated cells per slide was calculated from at least 15 random 100 × fields (**Fig 6*C***). The average percentage of differentiated cells was 9% ± 2%. A similar number of differentiated cells was found after treatment with human IFN-β antibodies (11% ± 3%). The number of K1-positive cells was significantly reduced by treatment with murine IFN-β antibodies (1% ± 1%). When murine IFN-β was depleted from the medium containing 0.12 mM Ca^{2+}, the number of differentiated cells was reduced to that found in nondifferentiating medium (0.05 mM Ca^{2+}) (compare **Fig 6*C*** with 0 h time point in **Fig 3**).

To determine whether the presence of IFN-β in the culture medium was sufficient to induce differentiation, we treated primary murine keratinocytes for 24 h with recombinant murine IFN-β. Exogenous IFN-β treatment did not induce the expression of the differentiation-specific antigens K1 and loricrin in keratinocytes incubated in nondifferentiating medium (0.05 mM Ca^{2+}). Moreover, exogenous murine IFN-β did not enhance expression of K1 or loricrin in cells growing in differentiating medium (**Fig 7**). Whether a longer exposure to IFN-β would have induced differentiation is unclear. In agreement with earlier experiments, TGF-β (1 ng per ml) did not affect differentiation (**Fig 7**, *lane 7*).

DISCUSSION

These results demonstrate that differentiated or nondividing murine and human epidermal cells express IFN-β *in vivo* and *in vitro*. The pattern of IFN-β protein expression was similar in both murine and human skin. Keratinocytes in the basal layer did not produce IFN-β, whereas those in the suprabasal layers did. The expression of IFN-β directly correlated with expression of the differentiation markers K1 (Roop *et al*, 1987) and loricrin (Bickenbach *et al*, 1995) and inversely correlated with PCNA (Coltrera and Gown, 1991; Wolf and Dittrich, 1992) and BrdU (Gray, 1985; Iatropoulos and Williams, 1996) staining. Murine keratinocytes whose growth was arrested by TGF-β or ara-C did not express the differentiation

markers but did express IFN-β, suggesting that the production of IFN-β by terminally differentiated cells was associated with cessation of proliferation.

IFN-β is a multifunctional regulatory cytokine that can directly inhibit proliferation of tumor cells of different histologic origins (Borden *et al*, 1984; Sica *et al*, 1989; Fukuzawa and Horikoshi, 1992; Johns *et al*, 1992; Gorlach *et al*, 1994). Recent studies indicate that IFN-β can also downregulate the expression of angiogenic molecules such as interleukin-8 (Oliveira *et al*, 1992, 1994; Singh *et al*, 1996), basic fibroblast growth factor (Singh *et al*, 1995; Dinney *et al*, 1998), and matrix metalloproteinases (Fabra *et al*, 1992; Gohji *et al*, 1994) that are necessary for tumor growth and metastasis (Fidler, 1995). The expression of IFN-β by tumor cells could restrict their proliferation and growth and, indeed, neither human nor transgenic mouse squamous cell carcinomas expressed significant levels of IFN-β.

The extent of angiogenesis is determined by the balance between positive and negative regulating molecules (Malhotra *et al*, 1989; Liotta *et al*, 1991; Fidler and Ellis, 1994; Iruela-Arispe and Dvorak, 1997). We have found that exposure of mice to UV irradiation induces epidermal hyperplasia in association with an increase in expression of positive angiogenic molecules basic fibroblast growth factor and a decrease in expression of negative angiogenic molecules (IFN-β) in epidermal keratinocytes (Bielenberg *et al*, 1998b). This imbalance favors cutaneous angiogenesis. As the hyperplasia subsides, IFN-β protein expression is restored, and cutaneous vascularization returns to normal (Bielenberg *et al*, 1996).

In this study, murine and human squamous cell carcinomas did not express significant levels of IFN-β protein, in agreement with earlier findings that cutaneous infantile hemangioma and cutaneous melanoma express positive angiogenic factors but not IFN-β (Bielenberg *et al*, 1998b; Wineland *et al*, 1998). The expression of IFN-β by differentiated normal epithelial cells may explain how these cells could suppress the early stages of neoplastic regression in stratified epithelium (Javaherian *et al*, 1998). The lack of IFN-β expression by dividing cells may also explain why IFN-β protein or RNA expression are difficult to detect *in vitro* (Fujisawa *et al*, 1997).

The expression of IFN-β by keratinocytes was directly correlated with growth arrest, which is associated with terminal differentiation (Friedman *et al*, 1982; Yarden *et al*, 1984; Resnitzky *et al*, 1986; Belhumeur *et al*, 1993). We base this conclusion on the results of *in vitro* studies showing that differentiated keratinocytes no longer divided and expressed IFN-β. Nondifferentiated keratinocytes whose growth was arrested by TGF-β or cytarabine (Weinberg *et al*, 1995), however, also produced IFN-β. Similar to the case in M1 myeloid cells (Resnitzky *et al*, 1986) or embryonal carcinoma cells (Belhumeur *et al*, 1993), neutralizing IFN-β in the culture supernatants (by using specific antibodies) inhibited terminal differentiation.

In summary, we show that nondividing, terminally differentiated epidermal cells (keratinocytes) produce IFN-β. Although the primary role of IFN-β in the outer epidermal layers is probably to defend against viral, bacterial, and parasitic infections (Dianzani, 1992; Bielenberg *et al*, 1998a), IFN-β is also an integral part of a complex cytokine network within the skin. The production of IFN-β by nondividing cells may inhibit unregulated cutaneous angiogenesis, which is associated with progressive growth of neoplasms (Fidler and Ellis, 1994; Fidler *et al*, 1998).

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