Normal Human Epidermis Contains an Interferon-like Protein

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Interferons (IFNs) are a family of glycoproteins presently classified as alpha, beta, or gamma on the basis of biological and physiochemical characteristics (42). IFNs were first identified by their anti-viral activity during studies of viral interference (21), but have since been widely recognized to inhibit proliferation of both normal and malignant cells in vitro (4, 17, 19) and are under investigation as anti-tumor drugs in several clinical trials (2). We have previously demonstrated that cultured human keratinocytes produce IFN of an unknown type in response to herpes virus infection in vitro (35). We and others have shown that addition of either IFN-alpha or IFN-beta to human keratinocytes in vitro profoundly and reversibly inhibits cell growth (28, 47) and increases terminal differentiation, as determined by the percentage of cells with cornified envelopes and by the detachment rate for cells from the colony surface (47).

It is known that human lymphoblastoid cell lines can liberate IFN in small quantity in the absence of overt stimulation (32) and that there are substantial antigenic differences between the spontaneously produced IFN and the virally induced IFN (7). Recently, Moore and co-workers reported that bone marrow stem cells produce IFN when stimulated by a specific growth factor known as colony-stimulating factor 1 or macrophage growth factor, and that the cellular response to this growth factor is further augmented if cultures are treated with anti-IFN antibodies (27). These combined findings suggest that IFN may function as a physiological regulator of cell growth in vivo with properties of a negative growth factor or chalone (20).

We now report that IFN or a closely related protein is present in the proliferative compartment of normal epidermis in the absence of viral infection and suggest that this protein may represent a physiological growth inhibitor in human skin. This protein binds polyclonal antibodies to recombinant human IFN-alpha that have been purified by passage over an affinity column (Sepharose coupled to the recombinant interferon). The binding is localized in the proliferative (basal) compartment of human epidermis in histological cross-sections of normal skin and in cultured keratinocyte colonies. Extracts prepared from healthy nonvirally infected keratinocyte cultures contained interferon activity as determined by viral plaque inhibition assay. Using the Western blotting technique column-purified antibodies and antisera to recombinant human interferon-alpha recognized a band of ~40 kD when reacted with both extracted keratinocyte proteins and recombinant human interferon-alpha standards, that gave in addition a band of ~20 kD. The above findings suggest that interferon or a closely related protein is present in the proliferative compartment of normal epidermis in the absence of viral infection and therefore may serve as a physiological modulator of epidermal growth.

Materials and Methods

Tissues and Cell Culture

Newborn foreskins or cutaneous biopsies of healthy adult volunteers were embedded and frozen in tissue Tek II O.C.T. (Lab Tek Products, Naperville, IL). Primary keratinocyte (30) and fibroblast (12) cultures were prepared from newborn foreskins as described. At confluence dishes were washed twice with 0.02% EDTA, incubated in 0.25% trypsin at 37°C, and disaggregated to form a single cell suspension. Keratinocytes or fibroblasts were inoculated either on glass coverslips or (keratinocytes only) on dishes coated with human fibronectin 10 μg/cm² (II). Cultures were maintained at 37°C in 8% CO₂ and provided three times weekly with serum-free medium 199 supplemented with 10 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), 10 ng/ml epidermal growth factor (Bethesda Research Laboratories,
Purification of Anti-recombinant Human IFN-Alpha Antibodies from Rabbit Sera

Purification of anti-recombinant human IFN-alpha antibodies from two rabbit sera was obtained by conventional affinity chromatography (36) using a column of Sepharose 4B coupled to recombinant human IFN-alpha. The column was prepared using 5 mg of pure recombinant human IFN-alpha dissolved in and displayed against 0.1 M NaHCO$_3$ buffer pH 8.3 containing 0.5 M NaCl. The IFN solution was added to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) and the coupling was carried out according to manufacturer's recommendations. Either IFN-alpha antiserum or preimmune serum (0.3-0.7 ml) was added to the column. The column was washed with 7 column vol of phosphate-buffered saline containing 0.02 M NaHCO$_3$. Elution was then performed with 0.1 M NaHCO$_3$ with 0.3 M NaCl. The eluates were screened for the presence of antibodies to recombinant human IFN-alpha by Ouchterlony agarose double diffusion analysis and antibody neutralizing bioassay (34). Antibody titers were 1.7-5.1 x 10$^6$ interferon neutralizing units/ml protein.

Immunofluorescence

6-μm sections of fresh frozen tissues or cells were incubated with immune rabbit serum, preimmune rabbit serum, column-purified antibodies from immune serum, column-purified antibodies from preimmune serum, or monoclonal antibodies. The second antibody used was fluorescein-tagged goat anti-rabbit IgG or goat anti-mouse IgG (CooperBiomedical, Inc., Malvern, PA) (9).

Immunoblotting

Confluent keratinocyte cultures were prepared in a small volume of 0.5% Nonidet P-40 in Tris-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (41). The extracted proteins and pure recombinant human IFN-alpha (2.5 μg of a standard preparation) were reduced and separated by 12% SDS PAGE, then electrophoretically transferred to nitrocellulose paper using a Bio-Rad transblot apparatus (Bio-Rad Laboratories, Richmond, CA) overnight at 4°C, 60 V in Tris-glycine buffer with 20% methanol (41). Antigens on the nitrocellulose paper were incubated with either IFN-alpha antiserum or preimmune serum at 1:250-1:500 dilution, column-purified IFN-alpha antiserum, or column-purified preimmune serum at 1:50-1:250 dilution, and monoclonal antibodies to IFN-alpha at 1:50-1:500 dilution. Specific binding of antibodies was identified by immunoperoxidase staining of the nitrocellulose paper strips.

IFN Activity of Keratinocyte Extracts

Interferon activity of keratinocyte extracts was determined quantitatively by reduction of cytopathic effect of vesicular stomatitis virus using a bovine kidney cell line of epithelial origin (34). The extract samples used to measure interferon activity were serially diluted and added to infected cells either immediately after viral inoculation or 24 h later. Cultures were incubated at 37°C until the virally infected control cells displayed a 100% cytopathic effect. The IFN titer was then read as the reciprocal of the extract dilution that protected 50% of the cell monolayer. A laboratory standard of IFN was included in all assays.

Results

Antibody Binding

Two sera obtained from rabbits immunized against recombinant human IFN-alpha produced staining of the epidermal basal layer in tissue cross-sections with occasional staining in the first few suprabasilar layers (Fig. 1 a), while control specimens incubated with preimmune serum were negative (Fig. 1 b). Three monoclonal antibodies to recombinant human IFN-alpha gave negative staining when reacted with cross-sections of normal skin.

To determine whether the IFN staining was retained in cultured epidermis, second passage human keratinocyte cultures grown under serum-free conditions (25) were either detached from the dish by the use of Dispase II (16) and frozen or grown on coverslips and fixed in -20°C acetone for 30 s (40). Immunofluorescent staining was present in the basal layer of stratified colonies in a pattern analogous to that observed in vivo (Fig. 2 a), and individual cells displayed bright cytoplasmic fluorescence (Fig. 2 b). Stratified colonies incubated with preimmune serum were negative (Fig. 2 c and d). Cultured epidermis inoculated with monoclonal antibodies failed to stain. Since both human and rabbit sera may contain antibodies to keratin proteins (22), we repeated these studies using cultures of rapidly proliferating human dermal fibroblasts. Fibroblasts incubated with immune sera also displayed bright fluorescence, suggesting but not proving that the staining pattern observed in the keratinocytes was not due to keratin.

To further exclude the possibility that the staining pattern was due to antibodies nonspecifically induced during the immunization procedure, the reactive rabbit anti-sera were applied to an IFN affinity column of Sepharose 4B coupled to recombinant human IFN-alpha (36). Human sera eluted from the IFN affinity column (specifically bound) and material in the column washes (nonbound) were separately concentrated and used in the above procedure. Skin sections reacted with the purified antibodies present in the concentrated eluant revealed the same fluorescent staining pattern observed with whole sera (Fig. 1 c), while the comparatively concentrated washes gave no staining whatsoever (Fig. 1 d). Similarly, cultured epidermis reacted with the purified antibodies present in the concentrated eluant revealed the same fluorescent pattern as whole sera (Fig. 1 e).
staining pattern observed with whole sera (Fig. 2 e and f), while the comparably concentrated washes again gave no staining.

**Interferon Activity in Keratinocyte Cell Extracts**

To determine whether the positive staining pattern was associated with the presence of biologically active IFN, cell extracts and conditioned medium from confluent keratinocyte cell cultures, nonconditioned medium, and extracting solution alone prepared as described (40) were analyzed for IFN activity in a cytopathic effect assay that could detect as little as 2 interferon neutralizing units/ml of IFN activity (34). IFN activity was found exclusively in the cell extracts and was titered to 38 interferon neutralizing units/ml in triplicate samples, ~0.4% of the peak values measured previously by a different methodology in the medium of comparably confluent keratinocyte cultures infected with herpes simplex virus (35).

**Characteristics of Interferon Present in Cultured Keratinocytes**

To determine the molecular weight of IFN present in cultured keratinocytes, confluent cultures were extracted as above, the proteins separated by SDS PAGE and transferred...
Figure 2. Binding of anti-IFN antiserum to stratified keratinocyte cultures. Antibodies from immune serum (a) and column-purified anti-IFN antibodies (e) bound to the basal cell layer (BCL) of cultured keratinocyte colonies sectioned vertically and displayed bright cytoplasmic fluorescence in the respective en face preparations (b and f). Antibodies from preimmune serum did not bind to vertically sectioned keratinocytes colonies (c) or to keratinocytes grown on coverslips (d). Bar, 5 μm.

to nitrocellulose paper (46), and the lanes reacted separately with either immune sera, column-purified anti-IFN antibodies from these sera, appropriate control sera, column-purified preimmune sera, or monoclonal antibodies to IFN. Antigen binding on nitrocellulose paper was identified by immunoperoxidase staining (41). Immune sera and the affinity column-adherent antibodies from these sera bound principally to an ~20-kD recombinant human IFN-alpha standard and, to a lesser degree, to its ~40-kD dimer (Fig. 3), as expected (31). Monoclonal antibodies bound to these bands as well (data not shown). In lanes containing cell extracts, antibodies derived from immune sera recognized only a protein of ~40 kD. Preimmune sera and affinity column eluants prepared from these sera did not bind either to
recombinant human IFN-alpha or to the ~40-kD protein in the cell extract (Fig. 3). The monoclonal antibodies did not bind to the cell extracts.

Discussion

We have shown that a protein cross-reacting with recombinant human IFN-alpha is present in the proliferative compartment of normal human epidermis in vivo and in vitro in the absence of viral infection and that extracts of cultured keratinocytes contain IFN-like anti-viral activity. The use of multiple newborn and adult skin donors effectively eliminates the possibility that clinically undetectable viral infection was responsible for these findings. The precise nature of the IFN-like substance in the basal layer of human epidermis remains unclear, however. Failure of three different anti-IFN-alpha monoclonal antibodies to bind this protein argues against its identity with this class of IFN, although masking of multiple antigenic sites within the keratinocytes, remains a possibility. Similarly, if the substance is indeed IFN-alpha, the absence of its usually predominant ~20-kD species must be explained, even though dimers of recombinant human IFN-alpha of ~40 kD are known to exist in vivo and in vitro and to persist under reducing conditions as in SDS PAGE (31). Present data do not allow us to distinguish between a modified monomeric form of IFN-alpha, a persistent dimer of the otherwise predominant monomer, and a previously unrecognized class of IFNs.

The epidermis constantly renews itself. In normal skin, however, the majority of the cells in the germinative basal compartment are blocked either in G1, G2, or G0, and do not cycle unless stimulated (18). The precise controls for epidermal cell proliferation in vivo are virtually unknown, although circumstances such as wounding, ultraviolet irradiation, certain disease states, and chronological aging have undeniable influences (1, 13). Substances reported to influence epidermal population dynamics in vitro include epidermal growth factor (33), calcium (3), cyclic nucleotides (15), prostaglandins (44), vitamin A (6, 37), and various tissue extracts (14, 29, 30), but their physiological roles in normal or diseased skin are speculative. The present data suggest that IFNs may have a physiological or therapeutic role in disorders such as psoriasis (8) that are characterized by reversible epidermal hyperplasia, accelerated epidermal turnover rate, and compromised keratinocyte differentiation.

Chalones were first conceptualized by Bullough in 1962 as tissue specific, species nonspecific substances that can inhibit cell division (5). Although tentatively identified in the epidermis (5, 26) and avidly investigated over many years, chalones have proven elusive (24, 26). Although IFNs do not satisfy all the original criteria for epidermal chalones (5), their demonstrated ability to inhibit growth of cultured keratinocyte profoundly and reversibly (28, 47) make them excellent candidates for assuming such a function in vivo. IFN-alpha is known to behave as a negative feedback inhibitor for bone marrow cells at least in vitro (27), and the presence of IFN in the amniotic fluid of pregnant women during the second and third trimesters in the absence of detectable viral infection (23) suggests that IFN may participate in the regulation of fetal development. The present report demonstrates that an IFN-like protein is constitutively present in the proliferative layer of the epidermis. Intuitively, one might expect a chalone to be present in the suprabasilar nonproliferative compartment of the epidermis. However, the existence of such a regulatory factor in the basal layer, within potentially dividing cells that under normal conditions are nevertheless noncycling, is also plausible. We suggest that the IFN-like protein observed in these studies may therefore serve as a chalone in this precisely regulated tissue.

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References

