

An Unusual Strain of Human Keratinocytes Which Do Not Stratify or Undergo Terminal Differentiation in Culture

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Abstract. We have characterized an unusual cell phenotype in third passage cultures of a human keratinocyte strain derived from newborn foreskin epidermis. The cells had the same DNA fingerprint pattern as the second passage, morphologically normal, keratinocytes; they formed desmosomes and expressed the keratin profile characteristic of normal keratinocytes in culture. However, unlike normal keratinocytes, the cells did not grow as compact colonies and did not stratify or undergo terminal differentiation, even after TPA treatment or suspension culture. For these reasons we named them ndk for "nondifferentiating keratinocytes." The ndk cells also differed from normal keratinocytes in that they did not require a feeder layer and were not stimulated by cholera toxin to proliferate.

The ndk cells had an absolute requirement for hydrocortisone and their growth rate was increased when epidermal growth factor was added to the medium. Although ndk failed to undergo terminal differentiation in culture, they were not transformed, since they were still sensitive to contact inhibition of growth, did not proliferate in soft agar, and had a limited lifespan in culture. The appearance of the ndk phenotype was correlated with a doubling of chromosome number and the presence of a 1p marker chromosome. We suggest that these cells are a useful experimental adjunct to cultures of normal keratinocytes, in which proliferation and terminal differentiation are tightly coordinated, because in ndk cells there appears to be a block in terminal differentiation.

A number of techniques have been developed that allow the growth of normal human epidermal keratinocytes in culture (reviewed by Fusenig, 1986). One widely used method is to grow the cells with a feeder layer of mouse 3T3 cells in medium supplemented with cholera toxin, hydrocortisone, and epidermal growth factor (EGF)¹ (Rheinwald and Green, 1975; Rheinwald, 1980). Under these conditions, the cells form stratified colonies and retain many of the properties of the tissue from which they were derived. Proliferation is restricted to the basal layer and cells that leave it enlarge and undergo terminal differentiation (reviewed by Green, 1980; Watt, 1988).

Strains of human keratinocytes can be grown for ~50 generations before they senesce (Rheinwald and Green, 1975). This contrasts with the observed properties of rodent keratinocyte cultures, in which spontaneous immortalization and transformation is a relatively frequent phenomenon (Sanford and Evans, 1982). The greater stability of the human genome has been invoked as an explanation for this difference (DiPaolo, 1983). Recently, however, two lines of spontaneously immortalized human keratinocytes have been reported (Baden et al., 1987; Boukamp et al., 1988). Both lines stratify and undergo terminal differentiation. One of the lines exhibits reduced growth factor dependency and can grow in soft agar (Boukamp et al., 1988), but neither line is tumorigenic.

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; ndk, nondifferentiating keratinocytes; PNA, peanut lectin.

We describe here an unusual human keratinocyte strain that arose in cultures derived from a normal newborn foreskin epidermis, prepared and cultured under the standard conditions used in this and other laboratories (Rheinwald and Green, 1975; Allen-Hoffman and Rheinwald, 1984; Watt, 1984). These cells differed in morphology and growth requirements from normal keratinocytes and failed to stratify or undergo terminal differentiation. Nevertheless, they expressed the profile of keratins characteristic of cultured keratinocytes and were not transformed. Karyotypic analysis showed that the appearance of this phenotype was correlated with a doubling of chromosome number. We discuss the implications of our findings for studies of keratinocyte terminal differentiation.

Materials and Methods

Cell Culture

Human keratinocytes were isolated from newborn foreskin epidermis as described by Rheinwald and Green (1975). Briefly, the dermis was removed by dissection and the epidermis cut into small pieces and digested with 0.125% trypsin, 0.01% EDTA, at 37°C. At 30-min intervals for up to 3 h released cells were collected and fresh trypsin/EDTA added. The cells were pooled and plated onto 3T3 cells that had been pretreated for 2 h with 4 µg/ml mitomycin C (Sigma Chemical Co., Poole, England), in medium composed of 1 part Ham's F12 and 3 parts DME, supplemented with 10% FCS (Sera-Laboratories, Crawley Down, Sussex, England), 1.8×10^{-4} M adenine, 5 µg/ml insulin (Sigma Chemical Co.), 0.5 µg/ml hydrocortisone

(Calbiochem, Cambridge Bioscience, Cambridge, England), 10^{-10} M cholera toxin (ICN Biomedicals, High Wycombe, Bucks, England), and 10 ng/ml human EGF (a generous gift from Dr. George-Nascimento, Chiron Corporation, Emeryville, CA) (FAD + FCS + HICE; Allen-Hoffman and Rheinwald, 1984).

The nondifferentiating keratinocytes (ndk) described in this paper arose from strain h keratinocytes. The ndk passage numbers given in the text count the primary strain h cultures as passage 1. The first passage to consist entirely of cells with the ndk phenotype was passage 4.

Normal keratinocytes used in comparative experiments were of strain c (Magee et al., 1987; Morrison et al., 1988). SVK-14 cells, a line of SV-40-transformed human keratinocytes, were obtained from Joyce Taylor-Papadimitriou (Taylor-Papadimitriou et al., 1982) and grown in FAD + FCS + HICE without 3T3 feeder cells.

All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. The medium was changed every 2–3 d and the cells were passaged when close to confluence, as described previously (Watt and Green, 1981).

Soft Agar Growth Assay

Triplicate 60-mm-diam bacteriological plastic dishes (Sterilin, Feltham, England) were seeded with 10^3 , 10^4 , or 10^5 cells in 1 ml of FAD + FCS + HICE containing 0.3% Noble agar (Difco Laboratories, Inc., Detroit, MI) over 4 ml of a 0.6% agar base (McPherson and Montagnier, 1964). The cultures were maintained at 37°C and fed each week with 1 ml of 0.3% agar medium. Colony formation was assessed by microscopical examination at 2 and at 4 wk.

Induction of Premature Terminal Differentiation

Premature terminal differentiation of normal keratinocytes was induced in two ways. In the first, cells were harvested and cultured in suspension in FAD + FCS + HICE supplemented with 1.45% methylcellulose (4,000 centipoises; Aldrich Chemical Co., Milwaukee, WI) for 3 d (Green, 1977). In the second method, adherent cells were treated for 3 d with 5×10^{-9} M 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Sigma Chemical Co.; Parkinson et al., 1983).

Karyotyping

Chromosome spreads were made from cells in log phase growth. Where necessary, 3T3 feeder cells were first removed by EDTA treatment. Fresh medium was added to the cultures and the cells were incubated with 0.5 µg/ml colcemid for 1–2 h, harvested, and subjected to hypotonic shock. Chromosome spreads were prepared and G banding carried out by standard methods (Worton and Duff, 1979).

Antibodies and Lectins

The following antibodies and lectins were used for indirect immunofluorescence or Western blotting. Mouse monoclonals: Vim 13.2, reactive with vimentin (P and S Biomedicals Ltd., Liverpool, England); AE-1, reactive with keratins 10, 14, 16, and 19 (Woodcock-Mitchell et al., 1982); LP34, reactive with keratins 10, 18, and complexes of keratins 5 and 14, 6 and 16 (Lane et al., 1985); LE61, reactive with keratin 18 (Lane, 1982); LLO01 reactive with keratin 14 (a generous gift from E. B. Lane, Imperial Cancer Research Fund). Mouse polyclonal: BL10, reactive with keratins 5 and 6 (a generous gift from E. B. Lane, CRF). Guinea pig polyclonal: anti-230/205, reactive with desmoplakins; (Cowin and Garrod, 1983; Watt et al., 1984). Rabbit polyclonals: anti-PNA-gp, reactive with the peanut lectin-binding glycoproteins of keratinocytes (Morrison et al., 1988); anti-involucrin (Dover and Watt, 1987). FITC-conjugated peanut agglutinin (PNA-FITC) was obtained from Vector Laboratories Inc. (Peterborough, England) and FITC-conjugated second antibodies were obtained from ICN Biomedicals.

Indirect Immunofluorescence

For indirect immunofluorescence cells were grown on glass coverslips, washed in PBS, and fixed as follows: 1:1 methanol/acetone for staining with antibodies to intermediate filaments; 10 min on ice in absolute methanol for staining with anti-230/205; 10 min in PBS containing 3.7% formaldehyde for staining with anti-PNA-gp; 10 min in PBS containing 3.7% formaldehyde followed by 10 min on ice in absolute methanol for staining with anti-involucrin.

Cells were incubated with the first antibody or PNA-FITC for 45 min at room temperature, washed extensively in PBS, incubated with the appropri-

ate FITC-conjugated second antibody as before, washed again in PBS, mounted in Gelvatol (Monsanto Co., St. Louis, MO), and examined using a Zeiss Axiophot microscope.

Transmission Electron Microscopy

Confluent cultures of ndk cells were fixed in 2.5% glutaraldehyde in 0.7 M cacodylate buffer, pH 7.3, 0.002 M calcium acetate for 2–4 h, postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h, and dehydrated sequentially in 70%, 90%, and absolute ethanol. Specimens were embedded in Araldite resin and sections were cut both parallel and perpendicular to the culture substrate using a Reichert OMCI4. Sections were stained with uranyl acetate and lead citrate, and viewed in a Zeiss EM10 Electron Microscope.

DNA Fingerprinting

DNA was prepared from passage 2 strain h keratinocytes and passage 10 ndk by standard procedures (Maniatis et al., 1982) and digested with Hinf I (Boehringer Corp., London, England) in the presence of 4 mM spermidine trichloride. Fragments were resolved on a 0.8% agarose gel, transferred to Hybond-N membrane (Amersham International, Amersham, England) and fixed by UV irradiation. Filters were prehybridized at 57°C in phosphate buffer (Church and Gilbert, 1984) and hybridized overnight at 57°C in the same buffer containing 1 ng/ml of a ³²P-labeled cDNA probe (specific activity ~10⁹ cpm/µg DNA) corresponding to a hypervariable sequence 3' to the human α-globin gene (Higgs et al., 1981). Filters were washed for 5 min each at 57°C in phosphate buffer and then in 1× SSC, 0.1% SDS, and autoradiographed for 1–7 d at –70°C.

Preparation of Cytoskeletons

Confluent cell cultures were treated with EDTA to remove 3T3 cells, rinsed in PBS, and lysed for 15 min on ice in 20 mM Tris-HCl, pH 7.6, containing 1% NP-40, 5 mM MgCl₂, and 50 µg/ml leupeptin (Sigma Chemical Co.). Aliquots containing equal amounts of protein were incubated for 15 min on ice with DNase I (Boehringer Corp.; specific activity 20 × 10³ U/ml: 1 µl used per 100 µg of lysate) and then precipitated with 5 vol of acetone for 1 h on ice. After centrifugation at 14,000 rpm for 2 min, the pellet was air-dried, and cytoskeletal proteins were prepared by sequential extraction with low salt (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 3 mM EDTA, and 1% Triton X-100) and high salt (10 mM Tris-HCl, pH 7.4, containing 1.5 M KCl, 3 mM EDTA, and 1% Triton X-100) buffers, the pellet being vortexed and then incubated in each buffer for 10 min on ice. Proteins insoluble in high salt buffer were resuspended in electrofocusing sample buffer (O'Farrell, 1975) and separated by NEPHGE two-dimensional electrophoresis.

Gel Electrophoresis

One-dimensional SDS-PAGE was carried out according to the method of Laemmli (1970).

Two-dimensional electrophoresis for analysis of keratins was performed according to the method of O'Farrell et al. (1977) using nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension, in the presence of 9.5 M urea (Achtstaetter et al., 1986).

Ampholines (pH range 3.5–10.0, LKB Instruments Inc., Bromma, Sweden) were used to form the pH gradient in 100 mm × 3 mm rod gels. Electrophoresis was carried out at 200 V for 10 min, 300 V for a further 10 min, and 400 V for 5 h. The pH gradient established was measured by cutting rods run with sample buffer alone into 0.5 cm segments, eluting each segment overnight with 1 ml of water, and measuring the pH of the eluant. Actin (4 µg/rod), BSA (2.5 µg/rod), and yeast phosphoglycerate kinase (2 µg/rod, all from Sigma Chemical Co.) were mixed with cytoskeletal preparations as internal standards.

Western Blotting

Proteins were transferred to nitrocellulose (0.45 µm pore size; Schleicher & Schuell Inc., Dassel, FRG) at 0.25 A for 2 h at 4°C, using a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Watford, England) and transfer buffer consisting of 25 mM Tris base, 92 mM glycine, and 20% methanol (Towbin et al., 1979). Nonspecific protein binding sites were blocked by overnight incubation at 4°C in PBS containing 0.05% Tween 20 (Batteiger et al., 1982). Blots were incubated with specific antibody for 1 h at room temperature with vigorous shaking, washed three times over 5 min in tap

water, shaken with a 1:1,000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (ICN Biomedicals Inc.) for 1 h and washed as above. Blots were briefly reblocked by 20 min incubation in PBS containing 0.1% BSA, 0.05% Tween 20, and bound antibody visualized by incubation of blots in PBS containing 1 μ g/ml 3,3'-diaminobenzidine (Sigma Chemical Co.), 2 μ l/ml hydrogen peroxide. Total protein on blots was subsequently stained with India Ink (Glenney, 1986). Keratins were identified according to the catalogue of Moll et al. (1982).

Immunoprecipitation of Involucrin

Normal strain c keratinocytes and ndk were incubated overnight in medium containing 50 μ Ci/ml [35 S]methionine (Amersham International; specific activity 30 Ci/mmol). Next day, cultures were washed in PBS and extracted for 15 min on ice in 50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, and 0.1% Triton X-100. Extracts were clarified by centrifugation at 14,000 rpm for 2 min and aliquots containing 500,000 cpm of TCA-precipitable material were incubated on ice for 2.5 h with 2 μ l of normal rabbit serum, or 2 μ l of anti-involucrin serum. 30 μ l of a 1:1 (vol/vol) suspension of protein A-Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) was then added, and samples mixed end over end for 30 min at 4°C. Beads and associated immune complexes were collected by centrifugation and washed once in PBS containing 0.5% Triton X-100, 0.1% SDS, once in PBS containing 0.5% Triton X-100 and 0.5 M NaCl, and twice more in the first buffer. Pellets were resuspended in SDS-PAGE sample buffer containing 100 mM dithiothreitol (DTT), and boiled for 3 min. Immunoprecipitated proteins were analyzed on a 7.5% SDS-PAGE gel. The gel was treated with "Amplify" (Amersham International), dried, and autoradiographed at -70°C.

Measurement of Cornified Envelope Formation

To measure the percentage of cells with cornified envelopes, cells were harvested, counted, washed in PBS, resuspended for 5 min in PBS containing 1% SDS and 20 mM DTT, recentrifuged, and counted in a haemocytometer under phase optics (Sun and Green, 1976). In some experiments, cultures were treated with 0.8 M NaCl for 6 h to induce envelope formation (Rice and Green, 1979; Green and Watt, 1982) before harvesting.

Results

Derivation of ndk Cells

2×10^6 keratinocytes were isolated from a newborn fore-skin epidermis and plated at 2×10^5 cells per 9-cm tissue culture dish on mitomycin C-treated 3T3 feeder cells. Over the next 10 d, colonies of morphologically normal keratinocytes grew to confluence. These were designated strain h. At this time, frozen cell stocks were made from 9 of the dishes and the cells in the tenth dish passaged. Second passage cells were still morphologically normal (Fig. 1 a), but 4–5 d after seeding the third passage, an additional cell type was discernable in the cultures, located at the margins of keratinocyte colonies (Fig. 1 b). These cells did not form compact

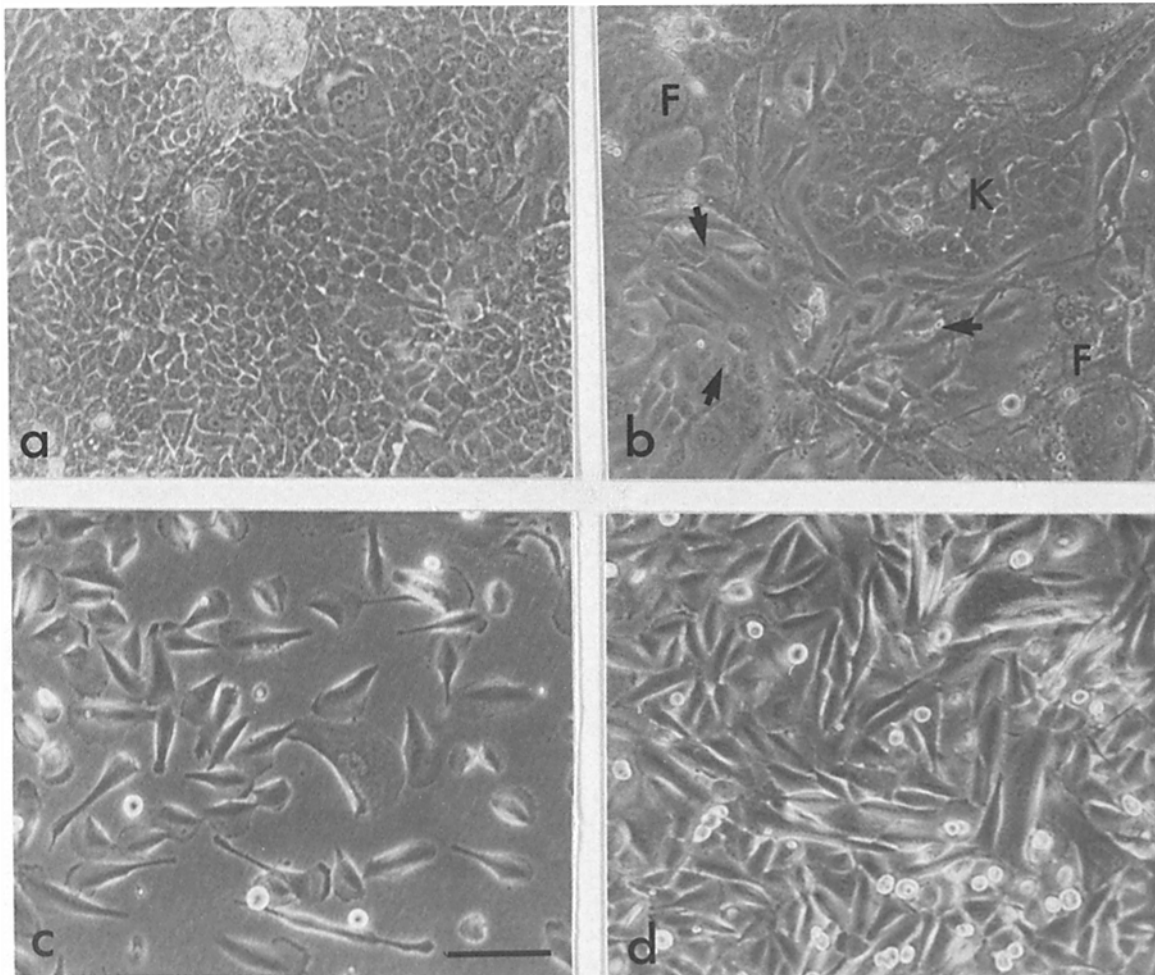


Figure 1. Identification of ndk cells. (a) Confluent strain h keratinocytes at passage 2. (b) Subconfluent strain h keratinocytes at passage 3. As well as keratinocyte colonies of normal morphology (K) surrounded by 3T3 feeder cells (F), a third cell type, ndk, can be seen (arrowed). (c) Pure population of ndk cells at passage 4, sparse culture. Note prominent ruffled membranes. (d) Dense ndk culture at passage 4. Bar, 50 μ m.

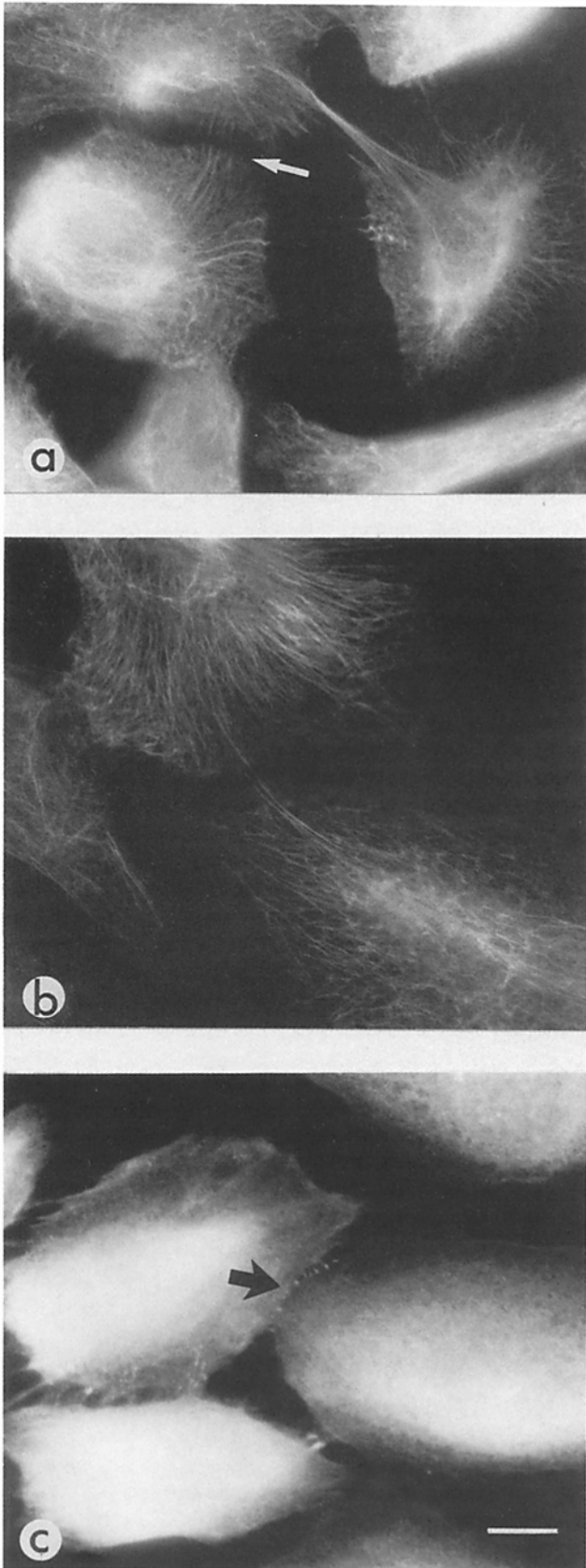


Figure 2. Identification of keratins and desmosomes in ndk cells by indirect immunofluorescence. *a* and *b* are keratin filaments stained with (*a*) LP34 and (*b*) LL001. Note alignment of filaments in areas of cell contact (*a*, arrowed). (*c*) Desmoplakins stained with anti-230/205. Arrow indicates desmosomes in regions of cell-cell contact. Bar, 10 μ m.

colonies and had a different morphology from normal keratinocytes with prominent ruffled membranes (Fig. 1 *c*). At high density, the cells formed a tighter sheet but there were no signs of stratification (Fig. 1 *d*). At this stage it was not clear whether the new cells had arisen through phenotypic conversion of the keratinocyte population or outgrowth of another cell type found in skin. As shown below, the former explanation appears to be correct, and we have therefore named the cells 'ndk,' for 'nondifferentiating keratinocytes.'

In time, the normal keratinocytes present in the third passage cultures were outgrown by the ndk cells. Unlike the 3T3 feeder cells, ndk cells could not be detached by EDTA treatment, but they were more trypsin sensitive than normal keratinocytes. Thus, by a combination of overgrowth and selective farming, it was possible to obtain a pure population of ndk cells. Subsequently, ndk cells were transferred to dishes with or without 3T3 feeder cells; it was apparent that they grew more successfully without the feeder layer, and so the cells have been grown subsequently without feeder support. The *in vitro* lifespan of ndk cells was similar to that of normal keratinocytes; the cells senesced at passage 18 when grown in FAD + FCS + HICE, without a feeder layer.

Epithelial Origin of ndk Cells

To investigate the origin of ndk cells, indirect immunofluorescence staining was carried out using antibodies directed against keratins. Positive staining was observed with LP34, an antibody that recognizes an epitope present on keratins of both stratified and simple epithelia (Fig. 2 *a*; Lane et al., 1985). The cells were not stained by LE61, which reacts with keratin 18, a keratin characteristic of simple epithelia (Lane, 1982; data not shown). Furthermore, the cells were stained positively by LL001, an antibody specific for keratin 14 (Leigh, I. M., P. E. Purkis, J. B. Steel, and E. B. Lane, manuscript in preparation) that is only present in cells of stratified squamous epithelia (Fig. 2 *b*).

The absence of simple epithelial keratins ruled out the possibility that ndk were Merkel cells or secretory cells of the sweat or sebaceous glands (Moll et al., 1982, 1984). The positive staining for keratin 14 indicated that the cells were keratinocytes, which could have originated from the epidermis or the junctional epithelium of sweat or sebaceous glands. Other cell types found in skin such as melanocytes or Langerhans cells do not contain keratin intermediate filaments (Wolff and Stingl, 1983). In addition, ndk did not stain with antibodies to factor VIII, and therefore were not of endothelial origin (data not shown). The morphology of ndk cells was similar to that of mesothelial cells; however, unlike ndk, mesothelial cells express keratin 18 and no keratin 14 (Connell and Rheinwald, 1983).

The coalignment of keratin filaments in adjacent cells (see Fig. 2 *a*) suggested that ndk cells possessed desmosomes, intercellular junctions characteristic of epithelial cells. Using a guinea pig antiserum raised against the desmoplakin proteins of the desmosomal plaque (Cowin and Garrod, 1983), positive immunofluorescent staining of ndk cells was observed at sites of cell contact (Fig. 2 *c*). The high level of cytoplasmic fluorescence may reflect the relatively low number of assembled desmosomes compared with normal keratinocytes (Watt et al., 1984).

To catalogue the keratins present in ndk, cytoskeletal extracts were made from eighth passage ndk cultures and from normal strain c keratinocytes. The extracts were run out on

two-dimensional gels, and individual keratins were identified by immunoblotting. Blots probed with monoclonal antibody AE-1 (Woodcock-Mitchell et al., 1982) indicated that keratins 14, 16, and 19 were present in both cell extracts, and that there were no alterations in the relative quantities of these keratins in ndk cells (Fig. 3, *a, b, c, and d*). A mouse polyclonal serum, BL10, detected keratins 5 and 6 in both ndk and strain c keratinocytes, although in ndk keratin 6 was present in lower amounts relative to keratin 5 (Fig. 3, *e and f*). Ndk did not contain detectable amounts of keratin 17, suggesting that the cells were not keratinocytes from sebaceous or sweat glands (Moll et al., 1982).

Since some epithelial cell lines coexpress keratin and vimentin intermediate filaments (Franke et al., 1979), we

also examined cytoskeletal extracts of ndk cells and normal keratinocytes for the presence of vimentin. Western blots of two-dimensional gels probed with a monoclonal antibody to vimentin revealed that passage 8 ndk cells contained vimentin, whereas normal strain c keratinocytes did not (Fig. 3, *a, b, g, and h*).

DNA Fingerprinting and Karyotyping of ndk Cells

To rule out the possibility that the appearance of ndk cells was due to contamination of the original keratinocyte cultures, the DNA fingerprints of passage 2 strain h keratinocytes and passage 10 ndk cells were compared using an α -globin 3' hypervariable region probe (Fowler et al., 1988).

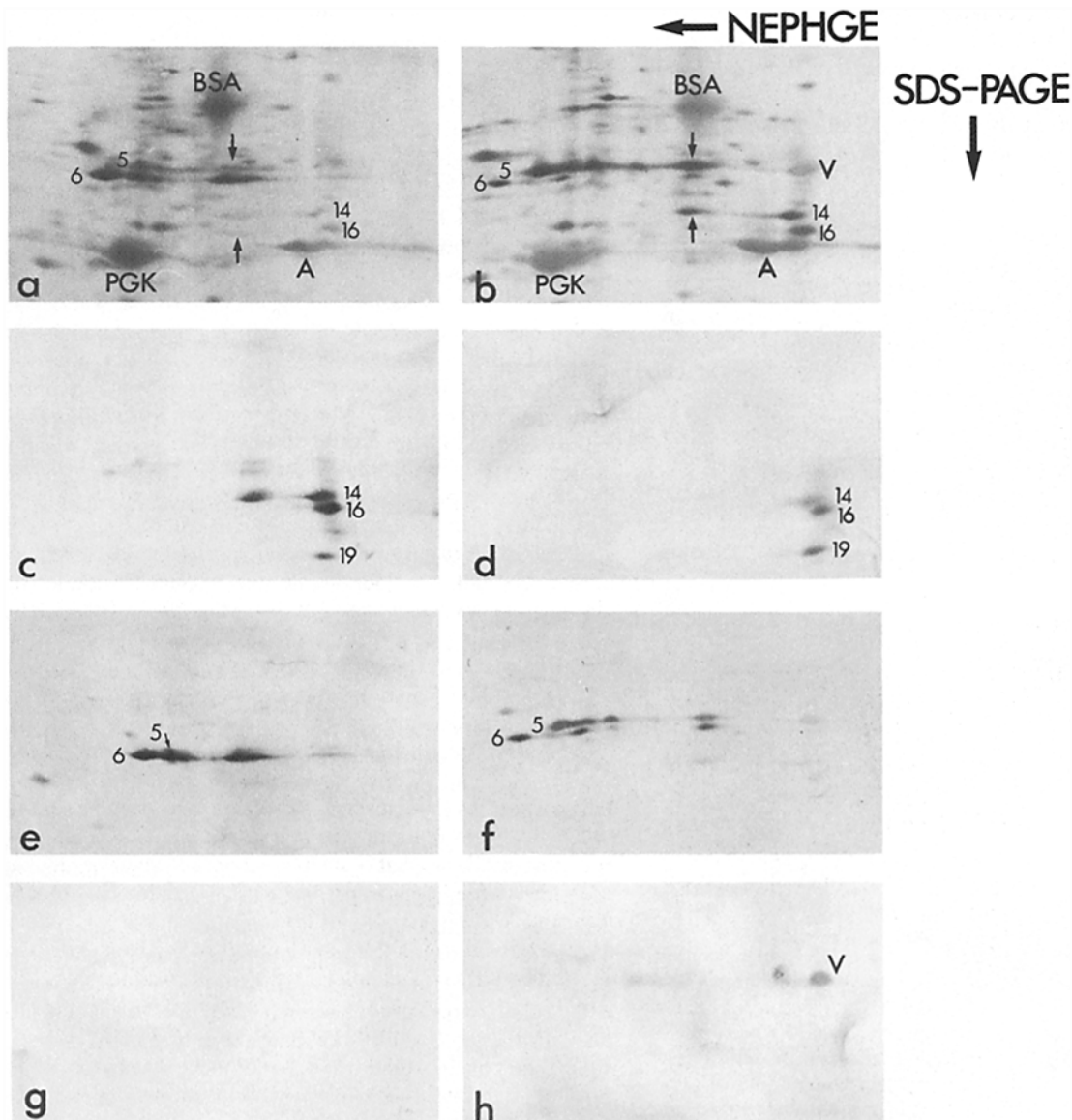


Figure 3. Identification of keratins in normal keratinocytes and ndk cells. Cytoskeletal extracts prepared from eighth passage strain c keratinocytes (*a, c, e, and g*) and ndk cells (*b, d, f, and h*) were analyzed by two-dimensional gel electrophoresis and immunoblotting. (*a and b*) Blotted proteins stained with India Ink. BSA, actin (*A*), and yeast phosphoglycerate kinase (*PGK*) were used as internal standards. Keratins are numbered according to the catalogue of Moll et al. (1982). Arrows indicate undissociated keratin complexes. *V*, vimentin. (*c-h*) Immunoblots probed with (*c and d*) AE-1, (*e and f*) BL10, and (*g and h*) VIM 13.2.

Unrelated human placental DNA was used as a control. Identical alleles were present in passage 2 strain h keratinocytes and passage 10 ndk cells (Fig. 4). Ethical considerations prevented us from obtaining a blood sample from the tissue donor, but the result demonstrated that both the morphologically normal keratinocytes and the ndk cells originated from the same individual.

To investigate whether the changes in phenotype described were associated with any gross chromosomal abnormalities, metaphase chromosome spreads were prepared from second passage strain h keratinocytes, ndk cells at passages 4 and 11, and also from normal strain c keratinocytes at passage 8. Strain c keratinocytes had a diploid karyotype with no obvious chromosomal abnormalities, as reported previously for other strains of keratinocytes in culture (Rheinwald and Green, 1975). ndk cells at passages 4 and 11 contained a hypotetraploid set of chromosomes; the G banding patterns were those of normal chromosomes (Fig. 5), and the only abnormality detected at either passage was a 1p chromosome, which was present in all metaphases. Spreads from passage 2 morphologically normal strain h keratinocytes contained a mixture of diploid (60%) and hypotetraploid (40%) metaphases, and no 1p chromosomes. Thus the dou-

bling of chromosome number preceded the changes in cell morphology (Table I).

Differentiated Properties of ndk Cells

In normal human keratinocyte cultures, the cells grow as stratified colonies and undergo terminal differentiation as they migrate through the outer layers (Green, 1980; Watt, 1988). Light microscopical examination of ndk cultures (Fig. 1) did not yield morphological evidence of terminal differentiation, such as cell enlargement (Watt and Green, 1981), or shedding of squames (Sun and Green, 1976). We therefore examined the ultrastructure of ndk cultures and analyzed the cells for specific markers of terminal differentiation.

Ultrastructure. Sections cut perpendicular to the culture substrate showed that the ndk cultures were only one cell layer thick (Fig. 6 a). In contrast, normal keratinocyte cultures typically consist of 6–8 layers at confluence (Rheinwald and Green, 1975). The cells contained prominent bundles of keratin filaments, abundant rough endoplasmic reticulum, and membrane-bounded vesicles. Desmosomes were observed at regions of cell contact, but there were fewer than in normal keratinocyte cultures (Fig. 6, b and c); (Watt et al., 1984; Magee et al., 1987). In some sections, the Golgi apparatus was clearly visible (Fig. 6 b).

Cornified Envelopes. In normal keratinocyte cultures, a small proportion of cells in the uppermost layers assemble a protein envelope under the plasma membrane. These cornified envelopes are insoluble in solutions containing SDS and reducing agents. Envelope assembly occurs when the envelope precursor proteins are cross-linked by a keratinocyte-specific transglutaminase in a reaction that requires calcium (Rice and Green, 1979). The percentage of cells "competent" to form cornified envelopes can be measured by raising the intracellular calcium ion concentration, for example by treatment with a high concentration of NaCl (Rice and Green, 1979).

Envelope formation was measured in newly confluent cultures of ndk and strain c keratinocytes under normal culture conditions and after treatment with 0.8 M NaCl. Salt treatment increased the percentage of strain c keratinocytes forming envelopes from 5 to 86%, indicating that most of the cells were competent to form envelopes. In contrast, ndk did not form cornified envelopes even after NaCl treatment (Table II).

Cornified envelopes form naturally when keratinocytes reach the final stages of terminal differentiation. Premature terminal differentiation, resulting in envelope assembly, can be induced by placing keratinocytes in suspension (Green, 1977) or treating them with TPA (Parkinson et al., 1983). We therefore exposed normal strain c keratinocytes and ndk to 5 nM TPA or suspended them in 1.45% methylcellulose for 3 d and measured cornified envelope formation. As shown in Table II, both treatments lead to a large increase in the percentage of normal keratinocytes with cornified envelopes, but neither treatment induced envelopes in the ndk cultures.

Involucrin. Immunoprecipitation and immunofluorescence techniques were used to examine ndk for the presence of involucrin, the major precursor protein of the cornified envelope (Rice and Green, 1979). Both techniques detected invo-

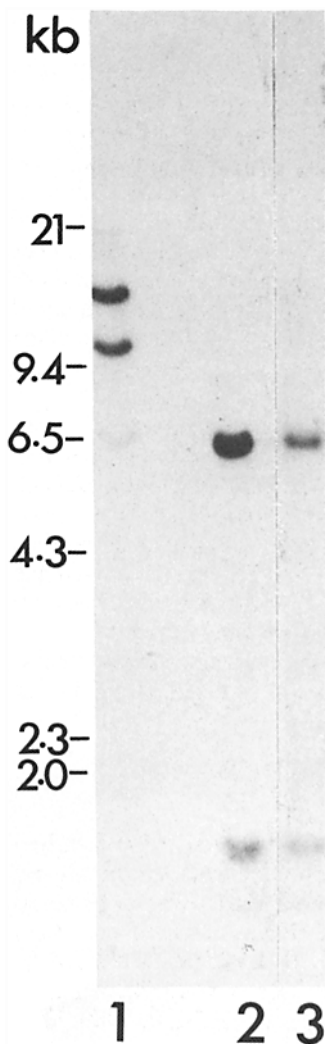


Figure 4. DNA fingerprinting of strain h keratinocytes and ndk cells. DNA from human placenta (lane 1), passage 10 ndk cells (lane 2), and passage 2 strain h keratinocytes (lane 3) hybridized with α -globin 3' HVR probe. Migration positions of λ phage fragments produced by digestion with Hind III and Eco RI are indicated.

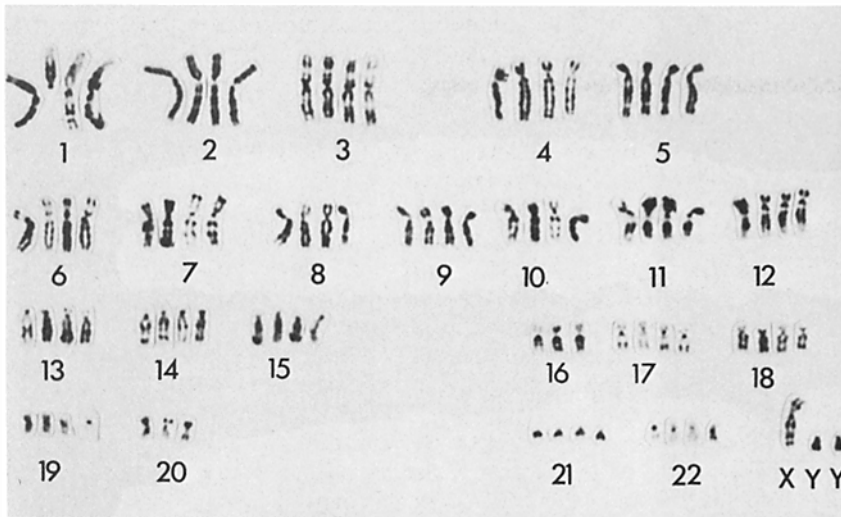


Figure 5. G-banded karyotype of ndk cells. Karyotype obtained from metaphase spreads of passage 4 cells.

lucrin in normal strain c keratinocytes, but not in ndk (Fig. 7 and results not shown). Furthermore, involucrin was not induced in ndk cells in response to TPA treatment or suspension culture (results not shown).

Peanut Lectin-binding Glycoproteins. Peanut lectin (PNA) binds more strongly to suprabasal, terminally differentiating, keratinocytes than to basal, proliferative keratinocytes, both in vivo and in vitro (Watt, 1983). Some epitope(s) of the PNA-binding glycoproteins is already expressed in the basal layer and can be detected with an antiserum to the glycoproteins, anti-PNA-gp (Morrison et al., 1988). ndk cell microvilli stained positively with anti-PNA-gp, but not with PNA-FITC (Fig. 8) and thus ndk resembled basal keratinocytes that had not begun to terminally differentiate.

Growth Properties of ndk Cells

As described above, ndk cells differed from normal keratinocytes in that they continued to proliferate when grown in the absence of a 3T3 feeder layer. Cell proliferation was examined under more stringent growth conditions by reducing the concentration of FCS from 10%, to 5, 2, or 0.5%, in the presence of the standard concentrations of added EGF, cholera toxin, insulin, and hydrocortisone. ndk cells in medium containing 10% FCS grew exponentially with a doubling time of 36 h and reached saturation density in 18 d. In medium containing 5 or 2% FCS, the cells attached and spread but did not grow for the first 10 d. After this lag period, they grew exponentially with a similar doubling time

to that of cells in 10% FCS, but had not reached saturation density by the end of the experiment (Fig. 9 A). Cells that grew in 2 or 5% FCS medium appeared morphologically similar to the total ndk population and stained positively with the antikeratin antibodies LP34 and LL001 (not shown). In medium containing 0.5% FCS, ndk cells attached but did not spread fully and did not proliferate.

To test the requirements of ndk for the growth factors normally added to keratinocyte culture medium (EGF, hydrocortisone, insulin, and cholera toxin) proliferation was examined in medium containing 10% FCS but lacking individual growth factors. In medium without added hydrocortisone the cells did not grow even though EGF, insulin, and cholera toxin were present; under these conditions the cells settled but did not spread well. Removal of cholera toxin or insulin did not alter the growth rate or saturation density of the cells. Omission of EGF caused the population doubling time to increase from 36 to 168 h even though the cells settled and spread normally (Fig. 9 B).

The capacity of ndk for anchorage independent growth was examined by suspending the cells in medium containing 0.3% agar. SVK14, an SV-40-transformed keratinocyte cell line (Taylor-Papadimitriou et al., 1982), was used as a positive control. After 2 wk, numerous colonies could be seen in the SVK14 cultures but no colonies were observed in ndk cultures even after 4 wk. Normal strain c keratinocytes were also unable to grow in soft agar (data not shown).

The grown properties of ndk were examined at several passage numbers and found to be stable throughout the lifespan of the cultures.

Table 1. Polyploidisation of ndk Cells

Passage number	Cell morphology	Ploidy of metaphases		Metaphases with 1 p marker
		2 n	4 n	
		%	%	%
2	Keratinocyte	59 (40-46)	41 (70-90)	0
4	ndk	0	100 (72-92)	100
11	ndk	0	100 (68-90)	100

Figures in brackets refer to range of chromosome numbers per metaphase.

Discussion

We have described a new phenotypic variant of human keratinocytes that arose in third passage cultures of foreskin keratinocytes from a healthy neonate, whose subsequent development has been normal. The cells, designated ndk, were unambiguously identified as keratinocytes on the basis of the keratins they expressed, since these were characteristic of cells from stratified epithelia (Fuchs and Green, 1980; Moll

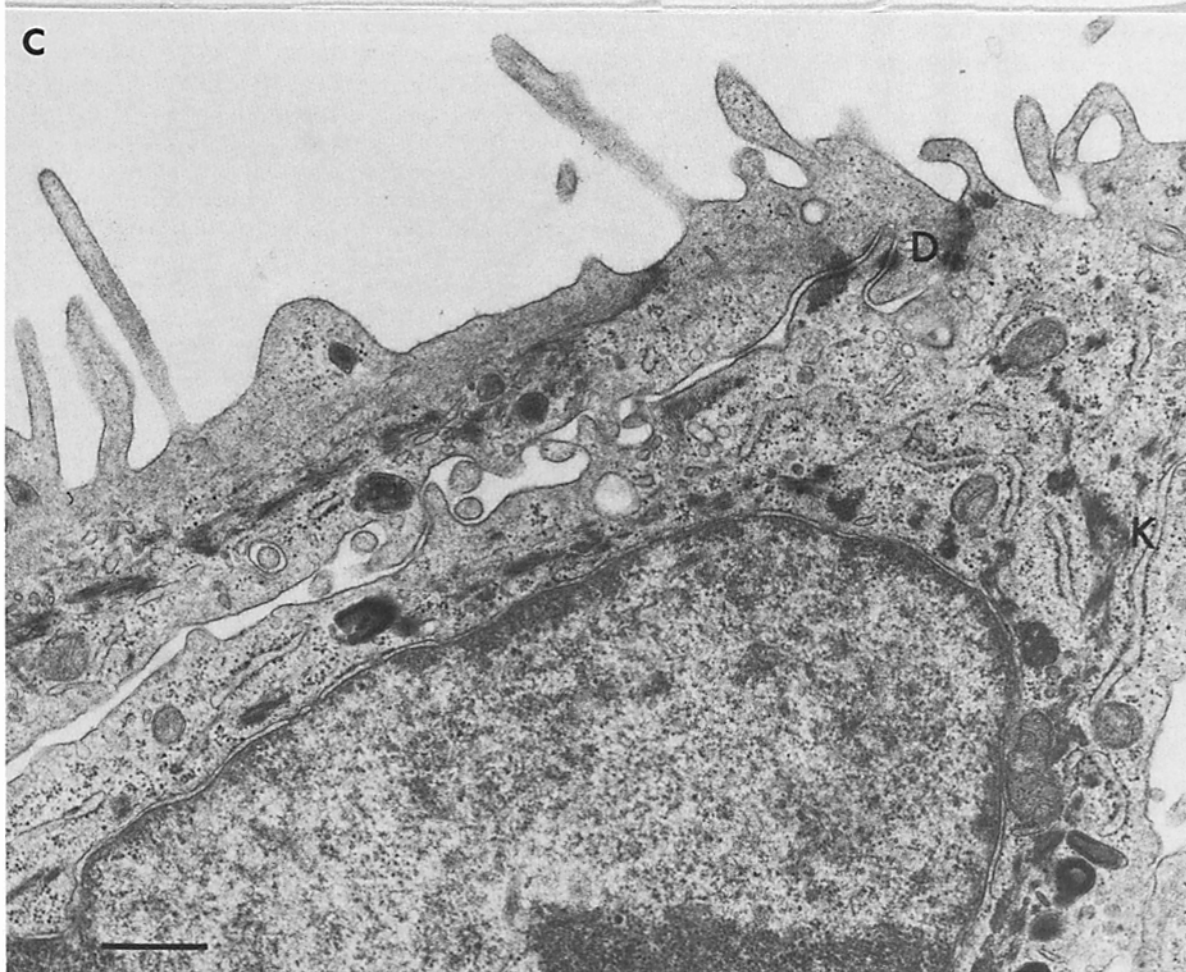
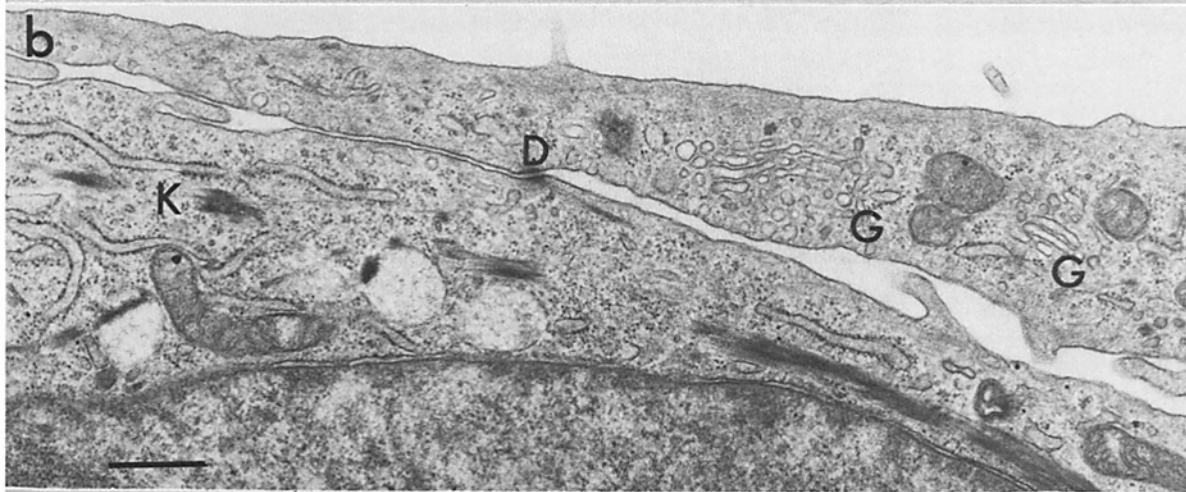
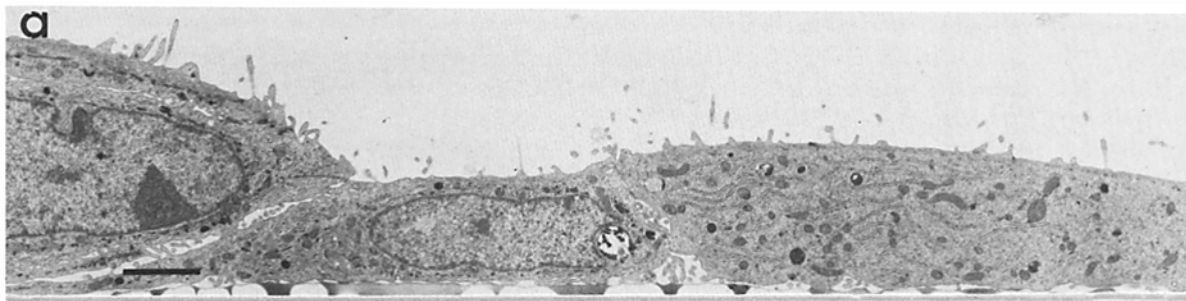


Table II. Induction of Cornified Envelope Assembly in Keratinocytes and ndk

Treatment	Percent of cells forming cornified envelopes	
	Strain c keratinocytes	ndk
Untreated	5 ± 1.2	0
0.8 M NaCl, 6 h	86 ± 3.5	0
5 nM TPA, 3 d	50 ± 2.8	0
Suspension cultures, 3 d	91 ± 4.1	0

Cells were trypsinised, counted, and scored for cornified envelope formation by treatment with PBS containing 1% SDS and 20 mM DTT. Figures quoted are mean values obtained from duplicate experiments ± range.

et al., 1982). The ndk cells and the second passage keratinocytes of normal morphology from which they were derived had identical alleles at a hypervariable locus and therefore originated from the same individual.

The ndk phenotype was not observed in passage 2 morphologically normal cultures, which contained a mixture of diploid and near tetraploid metaphases. It is possible that the ndk phenotype, both in vivo and in vitro, was suppressed by an excess of normal keratinocytes. Spreads from ndk cells (passage 4 onwards) were all hypotetraploid and all contained a 1p marker chromosome. Tetraploidisation, most likely through failure of cytokinesis, may have taken place in a large proportion of the cells in the first or second passage cultures, or could have occurred as a clonal event before or after the cells were isolated from the epidermis. The appearance of the marker chromosome argues for a clonal origin of cells with the ndk phenotype; since these cells could not be detected in passage 2 cultures the acquisition of the 1p marker may have conferred a selective growth advantage.

It is interesting that in all the cultures derived from different frozen stocks of passage 2 strain h keratinocytes, cells of ndk morphology have appeared at the third passage and overgrown the normal keratinocytes. In contrast, other keratinocyte strains that have been isolated and examined at an equivalent or greater passage number remain diploid (Rheinwald and Green, 1975; and our observations). Of the two spontaneously immortalized human keratinocyte lines that have been described, one is hypotetraploid (Boukamp et al., 1988) and the other is diploid with a trisomy of chromosome 8 (Baden et al., 1987).

The ndk cells did not undergo terminal differentiation in culture. They did not stratify; they did not express involucrin or bind PNA on their microvilli; and they did not assemble cornified envelopes, even after treatment with 0.8 M NaCl. In contrast to normal keratinocytes, terminal differentiation of ndk could not be induced by TPA treatment or suspension culture. In these respects ndk were unlike the spontaneously immortalized human keratinocyte lines, which retain the ability to stratify and undergo terminal differentiation (Baden et al., 1987; Boukamp et al., 1988). The lack of terminal differentiation in ndk cultures is all the more striking when

Figure 6. Ultrastructure of ndk cells examined by transmission electron microscopy. (a) Section perpendicular to dish surface showing that the cells grew as a monolayer. (b and c) Sections parallel to dish surface. Note keratin filaments (K), Golgi complex (G), and desmosomes (D). Bars: (a) 25 µm; (b and c) 0.5 µm.

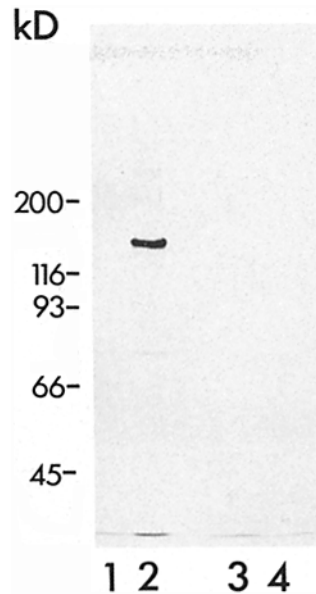


Figure 7. Immunoprecipitation of involucrin. [³⁵S]Methionine-labeled cell extracts of strain c keratinocytes (lanes 1 and 2) and ndk (lanes 3 and 4) immunoprecipitated with normal rabbit serum (lanes 1 and 3) or anti-involucrin serum (lanes 2 and 4).

one considers that even SV-40-transformed human keratinocytes and most lines derived from squamous cell carcinomas retain a limited capacity for terminal differentiation in culture (Banks-Schlegel and Howley, 1982; Steinberg and Defendi, 1979; Taylor-Papadimitriou et al., 1982; Rheinwald and Beckett, 1980). A subpopulation of normal human keratinocytes that do not terminally differentiate in response to TPA or suspension culture have been identified and it has been proposed that these may be stem cells (Parkinson et al., 1983; Hall, P. A., and F. M. Watt, unpublished observations). Since ndk cells appeared to be locked into the basal keratinocyte phenotype and did not undergo terminal differentiation they may have some of the characteristics of stem cells; however, their karyotypic abnormalities must limit their usefulness as a stem cell model.

The ndk cells resembled normal keratinocytes in their ability to grow in 2% FCS (Rheinwald, J. G., personal communication). However, some of their other growth properties, including feeder independence, were strikingly different. One function of the 3T3 feeder layer is to deposit an extracellular matrix on which keratinocytes attach and proliferate (Alitalo et al., 1982); our preliminary observations suggest that ndk cells secrete more fibronectin than normal keratinocytes, and this may partly explain why they did not require feeder cells. Like normal keratinocytes, ndk required hydrocortisone for growth; however, unlike the normal cells their proliferation was not stimulated by cholera toxin (Green, 1978). EGF prolongs the lifespan of normal keratinocytes without affecting their growth rate (Rheinwald and Green, 1977), but the growth rate of ndk was greatly increased in the presence of EGF. Taken together, these results may reflect alterations in extracellular matrix production, in growth

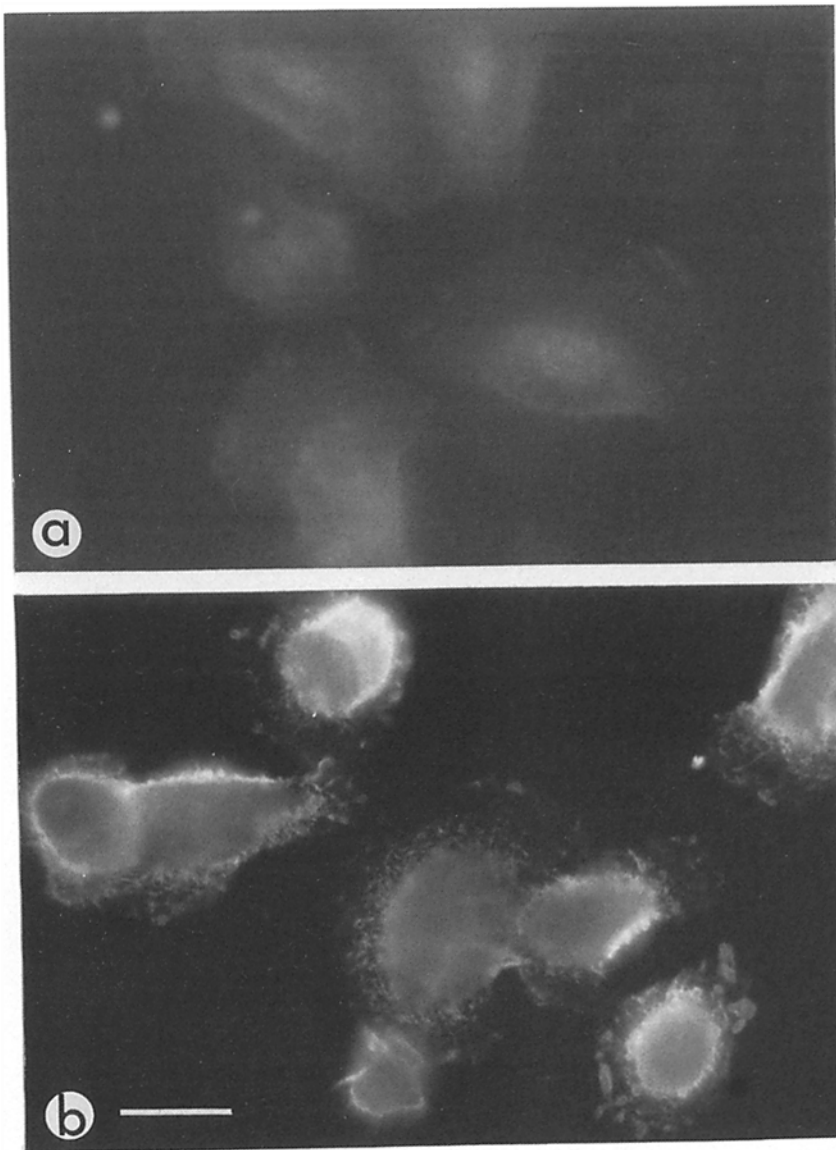


Figure 8. Indirect immunofluorescent staining of surface glycoproteins of ndk. Cells stained with PNA-FITC (a) or anti-PNA-gp (b). Bar, 10 μ m.

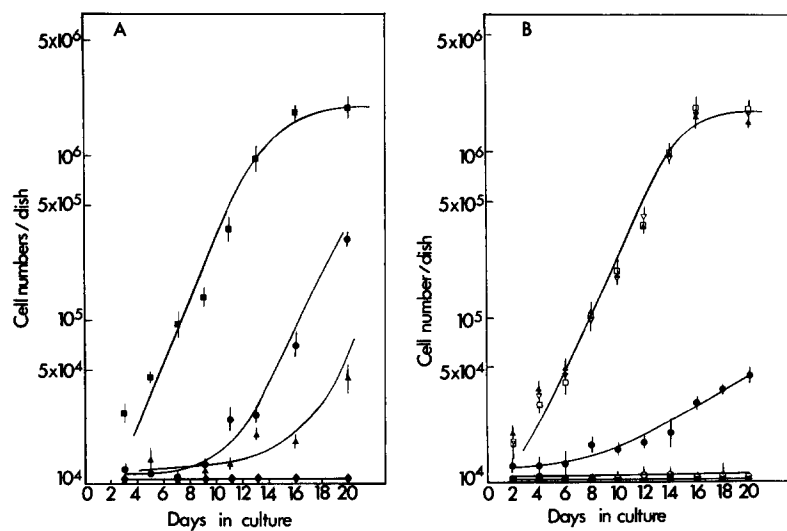


Figure 9. Growth properties of ndk cells. 10⁴ cells were plated per 60-mm dish. Three dishes were harvested per experiment on each day and each point is the mean of duplicate experiments. Error bars represent standard deviations. (a) Serum dependence. Cells were plated in FAD + HICE containing 10 (■), 5 (●), 2 (▲), or 0.5% (◆) FCS. (b) Growth factor dependence. Cells were plated in FAD + 10% FCS + HICE (▽), medium lacking insulin (▲), cholera toxin (□), EGF (●), hydrocortisone (○), or all four growth factors (■).

factor receptors, or intracellular signalling pathways, and this is currently under investigation.

Although ndk were unable to undergo terminal differentiation, they did not appear to be transformed. Their population doubling time was similar to that of normal keratinocytes; they exhibited contact inhibition of growth and could not grow in suspension. Furthermore, their lifespan was similar to that of normal human keratinocytes in culture. They may therefore provide an exception to the general observation that a break in differentiation is correlated with transformation and neoplastic progression (Harris, 1985; Klein, 1987; Bodmer, 1988).

In summary, we have identified and characterized an unusual keratinocyte phenotype that arose under our standard conditions for culturing keratinocytes from normal human epidermis. The cells were unable to terminally differentiate or to stratify in culture and differed in their growth properties from normal keratinocytes. ndk cells therefore provide a useful experimental adjunct to the normal cultures for investigating the factors that regulate keratinocyte proliferation and terminal differentiation.

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References

- Achtstaetter, T., M. Hatzfeld, R. A. Quinlan, D. C. Parmelee, and W. W. Franke. 1986. Separation of cyokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. *Methods Enzymol.* 134:355-371.
- Alitalo, K., E. Kuismanen, R. Myllyla, U. Kiistala, S. Askoseljavaara, and A. Vaheri. 1982. Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells. *J. Cell Biol.* 94:497-505.
- Allen-Hoffman, B. L., and J. G. Rheinwald. 1984. Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture. *Proc. Natl. Acad. Sci. USA.* 81:7802-7806.
- Baden, H. P., J. Kubilus, J. C. Kvedar, M. L. Steinberg, and S. R. Wolman. 1987. Isolation and characterization of a spontaneously arising long lived line of human keratinocytes (NMI). *In Vitro Cell. Devel. Biol.* 23:205-213.
- Banks-Schlegel, S. P., and P. M. Howley. 1982. Differentiation of human epidermal cells transformed by SV-40. *J. Cell Biol.* 96:330-337.
- Batteiger, B. W., J. Newhall, and R. B. Jones. 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *J. Immunol. Methods.* 55:297-307.
- Bodmer, W. 1988. Somatic cell genetics and cancer. *Cancer Surv.* 7:239-250.
- Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N. E. Fusenig. 1988. Normal keratinisation in a spontaneously immortalised aneuploid human keratinocyte cell line. *J. Cell Biol.* 106:761-771.
- Church, G. M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* 81:1991-1995.
- Connell, N. D., and J. G. Rheinwald. 1983. Regulation of the cytoskeleton in mesothelial cells: reversible loss of keratin and increase in vimentin during rapid growth in culture. *Cell.* 34:245-253.
- Cowin, P., and D. R. Garrod. 1983. Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity. *Nature (Lond.).* 302:148-150.
- DiPaolo, J. A. 1983. Relative difficulties in transforming human and animal cells in vitro. *J. Natl. Cancer Inst.* 70:3-8.
- Dover, R., and F. M. Watt. 1987. Measurement of the rate of epidermal terminal differentiation: expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques. *J. Invest. Dermatol.* 89:349-352.
- Fowler, S. J., P. Gill, D. J. Werrett, and D. R. Higgs. 1988. Individual specific DNA fingerprints from a hypervariable region probe: α -globin 3' HVR. *Hum. Genet.* 79:142-146.
- Franke, W. W., E. Schmid, S. Winter, M. Osborn, and K. Weber. 1979. Widespread occurrence of intermediate-sized filaments of the vimentin type in cultured cells from diverse vertebrates. *Exp. Cell Res.* 123:25-46.
- Fuchs, E., and H. Green. 1980. Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell.* 19:1033-1042.
- Fusenig, N. E. 1986. Mammalian epidermal cells in culture. In *Biology of the Integument*. Vol. 2. J. Bereiter-Hahn, A. G. Matoltsy, and K. S. Richards, editors. Springer-Verlag, Berlin. 409-442.
- Glenney, J. 1986. Antibody probing of Western blots which have been stained with India Ink. *Anal. Biochem.* 156:315-319.
- Green, H. 1977. Terminal differentiation of cultured human epidermal cells. *Cell.* 11:405-416.
- Green, H. 1978. Cyclic AMP in relation to proliferation of the epidermal cell; a new view. *Cell.* 15:801-811.
- Green, H. 1980. The keratinocyte as differentiated cell type. *Harvey Lect.* 74:101-139.
- Green, H., and F. M. Watt. 1982. Regulation by vitamin A of envelope cross-linking in cultured keratinocytes derived from different human epithelia. *Mol. Cell. Biol.* 2:1115-1117.
- Harris, H. 1985. Suppression of malignancy in hybrid cells: the mechanism. *J. Cell Sci.* 79:83-94.
- Higgs, D. R., S. E. Y. Goodbourn, J. S. Wainscoat, J. B. Clegg, and D. J. Weatherall. 1981. Highly variable regions of DNA flank the human α -globin gene. *Nucleic Acids Res.* 9:4213-4224.
- Klein, G. 1987. The approaching era of the tumor suppressor genes. *Science (Wash. DC).* 238:1539-1545.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.
- Lane, E. B. 1982. Monoclonal antibodies provide specific molecular markers for the study of epithelial tonofilament organization. *J. Cell Biol.* 92:665-673.
- Lane, E. B., J. Bartek, P. E. Purkis, and I. M. Leigh. 1985. Keratin antigens in differentiating skin. *Ann. NY Acad. Sci.* 455:241-258.
- MacPherson, I., and L. Montagnier. 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. *Virology.* 23:291-294.
- Magee, A. I., N. A. Lytton, and F. M. Watt. 1987. Calcium-induced changes in cytoskeleton and motility of cultured human keratinocytes. *Exp. Cell Res.* 172:43-53.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
- Moll, R., W. W. Franke, D. L. Schiller, B. Geiger, and R. Krepler. 1982. The catalogue of human cyokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell.* 31:11-24.
- Moll, R., I. Moll, and W. W. Franke. 1984. Identification of Merkel cells in human skin by specific cyokeratin antibodies: changes in cell density and distribution in fetal and adult plantar epidermis. *Differentiation.* 28:136-154.
- Morrison, A., S. Keeble, and F. M. Watt. 1988. The peanut lectin-binding glycoproteins of human epidermal keratinocytes. *Exp. Cell Res.* 177:247-256.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133-1142.
- Parkinson, E. K., P. Grabham, and A. Emmerson. 1983. A subpopulation of cultured human keratinocytes which is resistant to the induction of terminal differentiation related changes by PMA. Evidence for an increase in the resistant population following transformation. *Carcinogenesis (Lond.).* 4:857-861.
- Rheinwald, J. G. 1980. Serial cultivation of normal human epidermal keratinocytes. *Methods Cell Biol.* 21A:229-254.
- Rheinwald, J. G., and M. A. Beckett. 1980. Defective terminal differentiation in culture as a consistent and selectable character of malignant human keratinocytes. *Cell.* 22:629-632.
- Rheinwald, J. G., and H. Green. 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinising colonies from single cells. *Cell.* 6:331-344.
- Rheinwald, J. G., and H. Green. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature (Lond.).* 265:421-424.
- Rice, R. H., and H. Green. 1979. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell.* 18:681-694.
- Sanford, K. K., and V. J. Evans. 1982. A quest for the mechanism of "spontaneous" malignant transformation in culture with associated advances in culture technology. *J. Natl. Cancer Inst.* 68:895-913.
- Steinberg, M. L., and V. Defendi. 1979. Altered pattern of growth and differentiation in human keratinocytes infected by simian virus 40. *Proc. Natl. Acad. Sci. USA.* 76:801-805.
- Sun, T.-T., and H. Green. 1976. Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. *Cell.* 9:511-521.
- Taylor-Papadimitriou, J., P. Purkis, E. B. Lane, I. A. McKay, and S. E. Chang. 1982. Effects of SV40 transformation on the cytoskeleton and behavioural properties of human keratinocytes. *Cell Differ.* 11:169-180.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some

- applications. *Proc. Natl. Acad. Sci. USA*. 76:4350-4354.
- Watt, F. M. 1983. Involucrin and other markers of keratinocyte terminal differentiation. *J. Invest. Dermatol.* 81:100S-103S.
- Watt, F. M. 1984. Selective migration of terminally differentiating cells from the basal layer of cultured human epidermis. *J. Cell Biol.* 98:16-21.
- Watt, F. M. 1988. The epidermal keratinocyte. *Bioessays*. 8:163-167.
- Watt, F. M., and H. Green. 1981. Involucrin synthesis is correlated with cell size in human epidermal cultures. *J. Cell Biol.* 90:738-742.
- Watt, F. M., D. L. Matthey, and D. R. Garrod. 1984. Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. *J. Cell Biol.* 99:2211-2215.
- Woodcock-Mitchell, J., R. Eichner, W. G. Nelson, and T-T. Sun. 1982. Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. *J. Cell Biol.* 95:580-588.
- Wolff, K., and G. Stingl. 1983. The Langerhans cell. *J. Invest. Dermatol.* 80 (Suppl.):17s-21s.
- Worton, R. G., and C. Duff. 1979. Karyotyping. *Methods Enzymol.* 58:322-344.