

Oxygen-Dependent Differentiation of Human Keratinocytes

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Oxygen is an essential micronutrient. Unlike many internal tissues, human epidermis obtains much of its oxygen supply from the atmosphere (21% oxygen), and it ordinarily experiences higher oxygen levels than internal tissues (estimated $\approx 5\%$). To test whether epidermal cell growth and differentiation depend upon this higher oxygen level, keratinocyte cultures were studied at 21, 5, and 2% oxygen concentrations. Compared to 21% oxygen, culture in 5% had little effect on growth but led to profound suppression of the differentiation program as assessed by expression of differentiation markers and formation of squames in the superficial layers. Culture in 2% oxygen reduced the growth rate as well as stratification and differentiation. In low-oxygen conditions, the cells exhibited increased colony-forming ability, consistent with a lower proportion of differentiated cells, and increased expression of vascular endothelial growth factor and cyclooxygenase-2. Growth in 21% oxygen led to higher levels of glutathione and expression of oxidant-responsive genes. Electrophoretic mobility supershift assay using an involucrin activator protein 1 (AP1) response element sequence revealed altered binding by proteins of the Jun and Fos families in nuclear extracts. The present data thus demonstrate oxygen-dependent differentiation in human keratinocytes, to which altered utilization of AP1 transcriptional response elements may contribute.

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INTRODUCTION

As an essential micronutrient for cells, oxygen has a central role in oxidative phosphorylation, is a required cofactor for many enzymatic reactions, and modulates signal transduction pathways. The responsiveness of cells to oxygen tension in tissue culture is a physiological factor now receiving considerable attention. Except for the airways, the partial pressure of oxygen in tissues internally is often estimated as $\approx 5\%$, near that of the venous blood supply (Widmaier *et al.*, 2006). This level is considerably below the 21% found in ambient air. As noted with adipose-derived stem cells as they undergo chondrogenesis (Wang *et al.*, 2005) and with lymphocytes (Atkuri *et al.*, 2005), growth and various differentiated functions often respond differently to changes in oxygen tension. Finding an appropriate oxygen level is important to model proper behavior *in vivo*, including preventing early differentiation in embryonic stem cells (Ezashi *et al.*, 2005). Unlike many internal tissues, however, the epidermis has direct access to atmospheric oxygen. Supporting early estimates that the epidermis can obtain the

oxygen it needs by diffusion from the air (Fitzgerald, 1957), direct flux measurements indicate that the lower epidermis experiences an oxygen level of approximately 17%, with the blood supply making only a small contribution (Stuker *et al.*, 2002). Hence, cell culture at ambient oxygen conditions appears physiologically relevant to epidermal cell types.

Chronic wounds of the skin in regions of poor blood supply (e.g., diabetic ulcers) have been proposed to be refractory to healing owing to insufficient oxygen availability. This view has led to treatments where patients breathe elevated oxygen concentrations (often hyperbaric) or where the wound is treated topically with elevated oxygen concentrations. Experiments have shown promise for healing of dermal wounds in animals by topical (Fries *et al.*, 2005) or, under conditions of physiological stress, with systemic oxygen treatment (Gajendrareddy *et al.*, 2005). Although numerous human clinical successes have been reported, the lack of controlled studies (Phillips, 2000) has hampered acceptance of these treatments as routine therapy (Feldmeier *et al.*, 2005; Roeckl-Wiedmann *et al.*, 2005). Interpretation of treatment results may be difficult because wound healing is a complicated process with various participating cell types, including neutrophils, macrophages, fibroblasts, and keratinocytes. Additionally, microorganisms harbored by skin wounds may provoke inflammatory responses resulting in altered tissue oxygenation (Howell-Jones *et al.*, 2005). Certain aspects of wound healing, including collagen synthesis and leukocyte generation of superoxide for antibacterial action and prevention of infection, require oxygen (Tandara and Mustoe, 2004). However, evidence suggests

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Abbreviations: AP1, activator protein 1; EMSA, electrophoretic mobility supershift assay; hEp, normal human epidermal cell; SCC9, squamous-cell carcinoma 9; SIK, spontaneously immortalized keratinocytes

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that low oxygen levels, as seen with occlusive dressings, may actually promote healing as well. For example, hypoxia can stimulate fibroblast proliferation (Dimitrijevic *et al.*, 1999), and also induce plasminogen activator (Daniel and Groves, 2002) to stimulate migration in keratinocytes (O'Toole *et al.*, 1997). Such findings provide a basis for use of topical negative pressure devices to enhance healing of chronic wounds (Shirikawa and Isseroff, 2005).

Human keratinocyte culture using the Rheinwald-Green 3T3 feeder layer system is of assistance in modeling physiological responses, and permits stratification and the orderly expression of differentiation markers, resembling the natural epidermis (Green, 1979). These features ordinarily are coordinated with departure of the cells from the mitotic pool, so that stratification and differentiation culminate in the formation of terminally differentiated squames in the most superficial strata. Previous investigations showed that enlargement of the cells during this process is strongly related to growth potential, with the smallest cells having the highest colony-forming ability and the largest cells having virtually none (Sun and Green, 1976; Barrandon and Green, 1985). Synthesis of the differentiation marker involucrin occurs only in the larger cells (Watt and Green, 1981), found in the superficial layers (Banks-Schlegel and Green, 1981). This model has proven quite useful in studying physiological factors that influence regulation of keratinocyte programming (Watt, 1989; Fuchs, 1990). Present experiments test whether human keratinocytes depend upon a high oxygen level for their proper function. Further characterization of these cells under low-oxygen conditions may give insight into the complex role that oxygen plays in the epidermis and in wound healing.

RESULTS

Culture of human epidermal cells at low oxygen levels led to obvious changes in the microscopic appearance of growing colonies and confluent cultures. To characterize these changes, cultures of normal human epidermal cells (hEp) and the minimally deviated SIK line of spontaneously immortalized keratinocytes were fixed and sectioned. hEp (Figure 1a-c) and SIK cultures gave similar results. Hematoxylin and eosin staining revealed that the stratification ordinarily observed at ambient oxygen (21%) was greatly attenuated at low oxygen (2%). This effect was pronounced in SIK cultures grown without EGF, as shown in Figure 1d-f, where small nucleated cells of the basal layer were most pronounced at 2% oxygen. Concomitant with reduced stratification, expression of involucrin was markedly reduced in low-oxygen conditions (Figure 1g-i), as a consequence of the lack of superficial cells that ordinarily display this differentiation marker.

The distribution of cell sizes was measured in trypsinized cultures. Cells in preconfluent cultures were at the low end of the natural size range (peak about 16 μm diameter) regardless of oxygen level in the incubator. After confluence, however, colonies of normal epidermal cells grown and maintained in 21% oxygen became more stratified, and large squames appeared in the superficial layers. Measurement of cell sizes

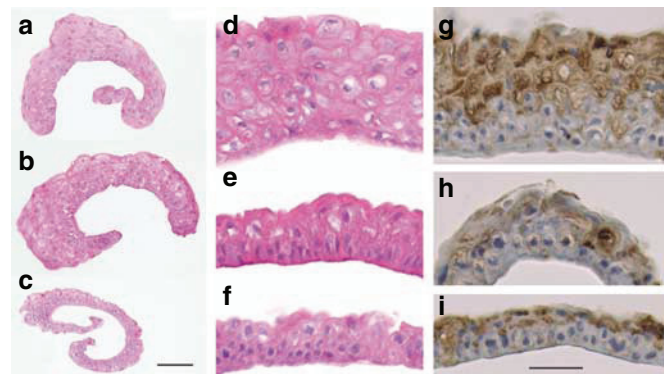


Figure 1. Cross-sections of colonies. Keratinocytes were inoculated at low density, equilibrated in 21% oxygen atmosphere for 48–72 hours, and transferred to the indicated oxygen atmosphere, where they were grown with or without EGF supplementation of the medium. The colonies were detached with dispase (Green *et al.*, 1979), formalin fixed, paraffin embedded, sectioned, and stained either with hematoxylin and eosin (a–f) or involucrin antiserum (g–i) as described previously (Phillips *et al.*, 2004). Illustrated are representative hEp colonies grown with EGF in (a) 21%, (b) 5%, or (c) 2% oxygen; sections of SIK colonies grown without EGF in (d) 21%, (e) 5%, or (f) 2% oxygen; sections of SIK colonies grown with EGF in (g) 21%, (h) 5%, or (i) 2% oxygen. Colonies were harvested at approximately 16 days. (a–c) Bar = 110 μm in panel (c), (d–i) Bar = 38 μm (i).

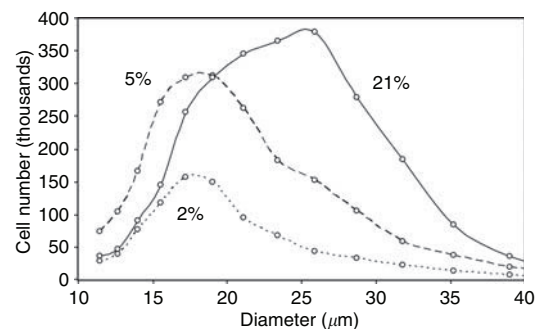


Figure 2. Effect of oxygen concentration on cell size distribution. One week after confluence, hEp cultures were trypsinized and sized using a Beckman Coulter Multisizer 3. Aliquots of cells from duplicate 6 cm cultures were counted twice and averaged. Shown are the total cell numbers per culture versus approximate diameters (provided by software), calculated from the measured volumes assuming spherical shapes.

at that time revealed a wide distribution. As seen in Figure 2, by a week after confluence, few of the cells remained in the low end of the size range, and the median diameter (25 μm) increased by approximately 50%. By contrast, the cells maintained in 5 or 2% oxygen remained small, and only a minor fraction was detected with increased diameter.

To find whether these changes in appearance were attributable to decreased growth at lower oxygen levels, growth rates were compared under the different conditions. As shown in Figure 3, with the SIK line, the rate of cell proliferation hardly differed between 21 and 5% oxygen, but it was noticeably slower in cultures grown in 2% oxygen.

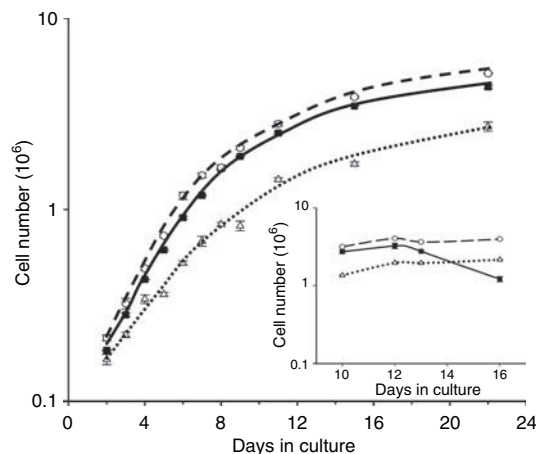


Figure 3. Cell growth versus oxygen concentration. SIK cultures maintained in the indicated oxygen atmosphere were washed twice with 0.5 mM EDTA in isotonic saline to remove the remaining 3T3 cells (Sun and Green, 1976), trypsinized, diluted into Isoton II diluent, and counted using a Z1 Particle Counter (Beckman Coulter, Fullerton, CA). Time points give the mean of three cultures counted in duplicate. Data shown are representative of three experiments. Inset: parallel experiment with cells inoculated at higher density, reaching confluence by day 10 and exhibiting prominent desquamation only at 21% oxygen. 21%, dashed; 5%, solid; 2%, dotted lines.

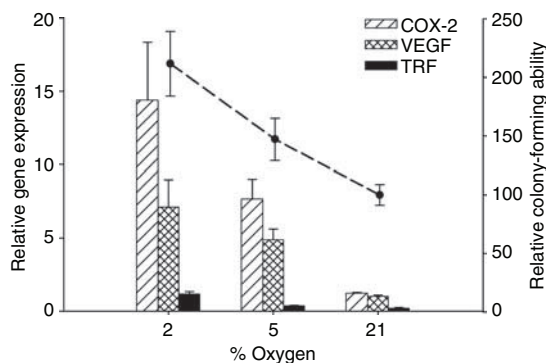


Figure 4. Properties of cultures as a function of oxygen concentration. hEp cultures were grown under the indicated oxygen concentrations. Expression of cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), and transferrin receptor (TFR) was measured by real-time PCR 18 days after confluence. Gene expression (bars) and colony-forming efficiency (dotted line) are given relative to 21% oxygen (1.0 and 100, respectively). For quantitation of colony formation (typically 6–7% at 21% oxygen), 700–1,000 cells were inoculated per 6 cm dish and were fixed and stained with rhodanile blue after 14–21 days.

When held at confluence in 21% oxygen conditions for 2 or more weeks, hEp and SIK cultures gradually lost differentiated cells by desquamation faster than they were replaced, leading to lower cell numbers. As shown, this phenomenon was not observed for confluent cultures maintained in 5 or 2% oxygen (Figure 3, inset).

In addition to reduced stratification and cell size observed in the lower oxygen conditions, other features of hEp cultures were indicative of reduced differentiation. As seen in Figure 4, cells grown in 2% oxygen had twice the colony-forming ability of those grown in 21% oxygen. Cultures grown in

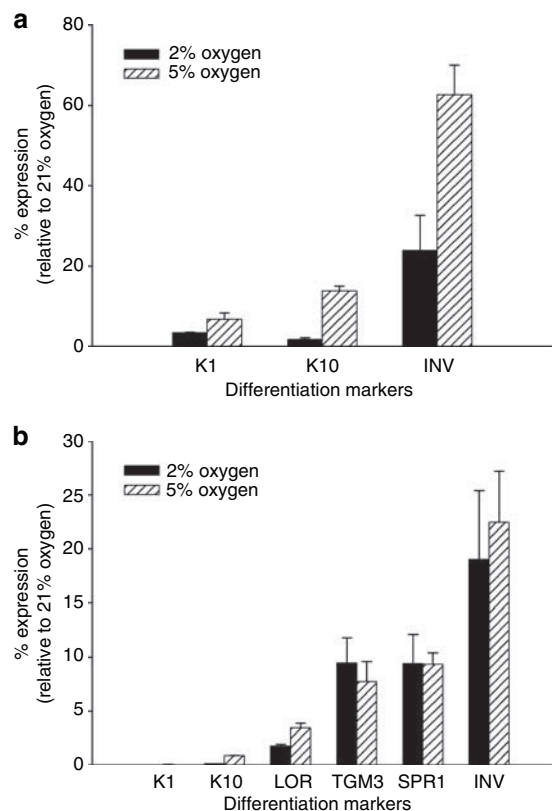


Figure 5. Expression of differentiation marker mRNAs as a function of oxygen tension. mRNA expression at the various oxygen levels was normalized to cultures grown in 21% oxygen (taken as 100%). (a) Keratins were measured 7 days and involucrin 14 days after confluence in hEp cultures. (b) The markers were measured 10 days after confluence in SCC9 cultures. K1, keratin 1; K10, keratin 10; LOR, loricrin; TGM3, epidermal transglutaminase 3; SPR, small proline-rich protein 1B (cornifin); INV, involucrin.

5% oxygen were intermediate in this respect. Similarly, the transferrin receptor, a marker for actively cycling cells (Webb *et al.*, 2004), was enriched in cells grown in low oxygen. Preliminary results indicate that the transcription factor basonuclin, confined primarily to the basal layer (Tseng and Green, 1994), was elevated as well (not shown). Most striking was the considerably higher expression in low-oxygen conditions of vascular endothelial growth factor and cyclooxygenase-2. The mRNA levels were approximately 7- and 15-fold higher, respectively, in 2% compared to 21% oxygen, and were also substantially elevated (7- and 5-fold) in 5% oxygen.

The effect on differentiation of growing keratinocyte cultures in low oxygen is illustrated in Figure 5a. Upon exposure of hEp to 2% oxygen, involucrin mRNA levels were only 25% those in 21% oxygen, whereas mRNA levels of keratins 1 and 10 were suppressed to less than 5% of those at the high-oxygen condition. Cultures grown in 5% oxygen exhibited intermediate expression levels of the markers. In an experiment with SIK, the influence of oxygen on involucrin content was essentially identical, where the levels of mRNA at 2 and 5% oxygen were 21 and 69%, respectively, of those

at ambient oxygen. The human cell line squamous-cell carcinoma 9 (SCC9), which expresses involucrin at moderate levels after confluence, was even more sensitive to the suppression of this differentiation marker than the SIK line. As shown in Figure 5b, involucrin expression was markedly reduced when the SCC9 cells were grown in 2 or 5% oxygen. A battery of other differentiation markers (keratin 1, keratin 10, small proline-rich protein 1, loricrin, transglutaminase type 3) displayed similar or even greater suppression.

To find whether the cell responses could be attributed to altered internal oxidation, the level of glutathione was measured. As shown in Figure 6a, the glutathione content of both SIK cultures at 5% oxygen was approximately half that at 21% oxygen. In experiments with SCC9 (not shown), the glutathione content at 2% was one-third and at 5% half that at 21% oxygen. As shown in Figure 6b, mRNA for the catalytic subunit of the rate-limiting enzyme in glutathione synthesis, glutamate-cysteine ligase, increased with oxygen level. Heme oxygenase-1 mRNA increased substantially as well. Preliminary experiments showed that NAD(P)H quinone oxidoreductase 1 and the modifier subunit of glutamate-cysteine ligase were also induced 60–70% at 21% oxygen compared to 2%. The genes encoding these enzymes are in the Nrf2 transcription factor response domain (Lee *et al.*,

2003), commonly used as an indicator of response to oxidative conditions.

Owing to the importance of activator protein 1 (AP1)-mediated gene expression in the differentiation of keratinocytes, the influence of oxygen tension on AP1 transcription factor interaction with the involucrin promoter was examined in the SCC9 carcinoma line. Cultures were examined as they neared confluence (little or no involucrin expression) and again a week after confluence when involucrin mRNA was accumulating. As shown in Figure 7, electrophoretic mobility supershift assays (EMSA) permitted detection of c-Fos, Fra-1, and JunB (but not c-Jun) in complexes with the functional AP1 response element from the proximal involucrin promoter. In preconfluent cultures under ordinary ambient conditions (21% oxygen), Fra-1 and JunB were readily detected in supershift assays, but a week after confluence only c-Fos was detectable. At 2% oxygen, Fra-1 and JunB were also observed in preconfluent cultures, but the pattern did not change after confluence. At 5% oxygen, JunB was observed in preconfluent cultures and persisted in the postconfluent samples. However, c-Fos was observed instead of Fra-1 at both time points. Thus, growth at lower oxygen levels perturbed transcription factor binding to the AP1 response element,

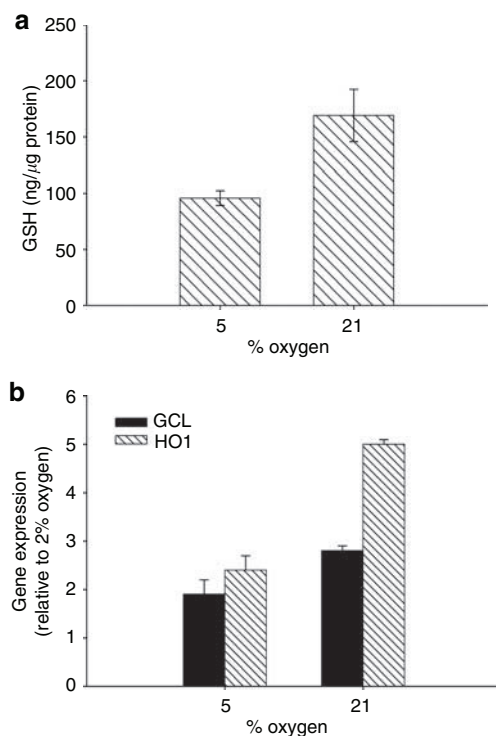


Figure 6. Evidence for oxidant activity as a function of oxygen tension. (a) SIK cultures were incubated for 3 days in 21 or 5% oxygen before measurement of glutathione content. (b) Cultures held at the indicated oxygen tension since confluence were harvested 10 days later for measurement of relative mRNA levels by real-time PCR; values are normalized to 1 for cultures held in 2% oxygen. HO1, heme oxygenase-1; GCL, glutamate-cysteine ligase (catalytic subunit).

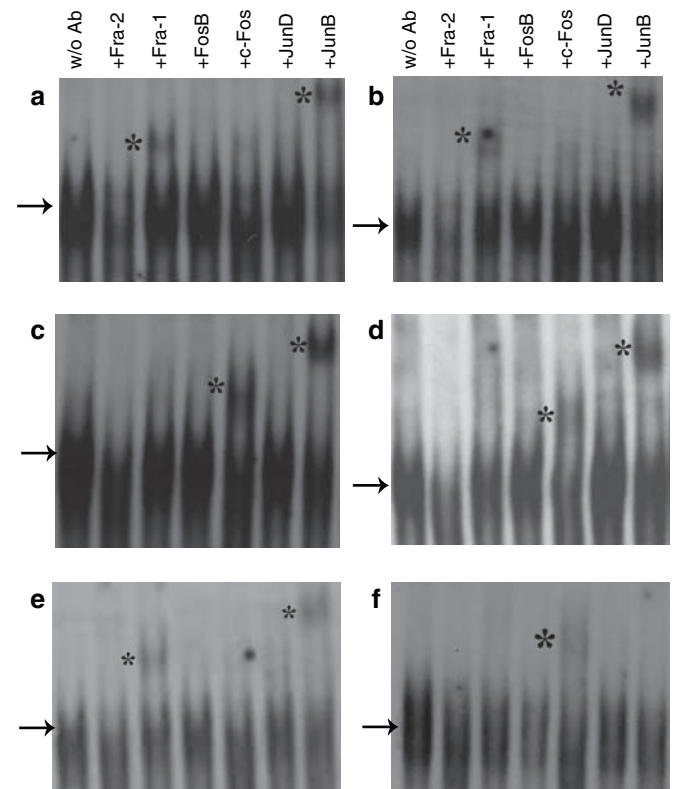


Figure 7. EMSA of AP1 transcription factors. Nuclear extracts were prepared from SCC9 cultures maintained in (a, b) 2%, (c, d) 5%, or (e, f) 21% oxygen harvested (a, c, e) a day before or (b, d, f) a week after confluence. Complex formation with an oligonucleotide from the proximal human involucrin promoter containing a functional AP1 response element was analyzed by EMSA, and constituents of the complex (arrows) were detected with antibodies for specific AP1 factors. The supershifted complexes are indicated by *.

where the presence of JunB was associated with a lack of involucrin expression.

DISCUSSION

The striking suppression by low oxygen growth conditions of keratinocyte stratification, enlargement, and differentiation suggests a profound role for oxidation in the programming of this cell type. A similar response is observed with mouse keratinocytes cultured in low-calcium medium, where stratification and differentiation are greatly reduced and growth is enhanced (Hennings *et al.*, 1980). Low-calcium medium also prevents stratification and reduces differentiation in human keratinocytes, although cell enlargement is not completely prevented (Watt and Green, 1982). Generation of reactive oxygen species within vascular smooth muscle (Touyz, 2005) and endothelial cells (Aley *et al.*, 2005) increases internal calcium levels, suggesting the involvement of oxidative controls of cellular calcium stores. We speculate that effects of low-oxygen conditions in keratinocytes resemble those in low-calcium medium because generation of reactive oxygen species is reduced. This is consistent with our observation that glutathione, needed for oxidant defense, was higher in the cultures held in high than in low oxygen. This response may be mediated at least in part by the Nrf2 transcription factor, which offers protection against oxidative conditions by stimulating, among other things, glutathione synthesis (Ishii *et al.*, 2000). Constituents of the non-phagocyte oxidase system, nicotinamide adenine dinucleotide phosphate (reduced form) oxidase, are expressed and functionally active in human skin (HaCaT) and gingival (GM16) keratinocyte lines (Chamulitrat *et al.*, 2004), making nicotinamide adenine dinucleotide phosphate (reduced form) oxidase a plausible candidate for oxygen sensing and oxygen-based regulation of growth and proliferation. Superoxide regulation of a variety of signaling pathways could be affected, particularly those involving redox-sensitive kinases and phosphatases (Chiarugi, 2005) and transcription factors such as AP1 (Sen and Packer, 1996).

A suppressive effect of chronic exposure to elevated oxygen levels on cell growth in culture has long been recognized (Balin *et al.*, 1984). Although intermittent exposure of keratinocytes in human skin equivalent cultures to high oxygen reportedly stimulates differentiation, as judged by stratification, with little effect on growth (Dimitrijevic *et al.*, 1999), higher than ambient oxygen levels are anticipated to induce oxidative stress in patients without antioxidant supplementation (Alleva *et al.*, 2005; Patel *et al.*, 2005). At the other extreme, human epidermal cells in preliminary work grow well in anoxic conditions, potentially with extended lifespan (Kino-oka *et al.*, 2005). In the present case, moderate reduction of oxygen to 5% had only a minor if any effect on keratinocyte proliferation, and reduction to 2% oxygen resulted in colonies that still grew progressively, albeit more slowly. Slower growth could result simply from lower efficiency of nutrient utilization by oxidative phosphorylation, but complex effects of oxidant activity on signaling pathways, including positive effects on growth factor receptors (Goldkorn *et al.*, 2005), suggest that subtler

effects are likely. The shift to a reduced degree of differentiation and lower stratification resulted in a reduced proportion of maturing cells, as reflected in lower differentiation marker expression and higher colony-forming ability. We conclude that use of elevated oxygen in chronic wound therapy does not alleviate an oxygen deficiency for keratinocytes. Moreover, the limited oxygen deprivation from topical negative pressure approaches (Shirikawa and Isseroff, 2005) would not be expected to be disadvantageous for the epidermis. In either case, effects of such therapies on processes in the dermis are likely to be more critical.

Neoplastic keratinocytes often display greater sensitivity than normal cells to alteration of differentiation by their microenvironment, and for this reason they can serve as useful models for mechanistic investigation (Rubin *et al.*, 1989). Involucrin is expressed in intermediate-grade SCCs of the uterine cervix (Azuma *et al.*, 2003) and in mouse xenografts of human head and neck squamous carcinoma A253 cells (Bhattacharya *et al.*, 2004) in regions exhibiting differentiated features but distant from the blood supply and hence anoxic. Such observations led to the hypothesis that involucrin expression is stimulated by hypoxia, a phenomenon reported in SCC9 cultures (Chou *et al.*, 2004). However, present results with SCC9 demonstrate striking suppression in culture of a battery of differentiation markers, including involucrin, under low-oxygen conditions. Lack of coincidence between areas of differentiation (where involucrin expression occurs) and hypoxia in SCCs is evident (Azuma *et al.*, 2003). Conditions leading to differentiation of the tumor cells *in vivo* are unknown, but marker expression under subsequent hypoxic conditions may be poorly reversible in the absence of cell division.

These findings emphasize the challenge of finding how oxygen levels influence the differentiation program and interact with other physiological influences. As utilization of AP1 response elements has been found critical for proper expression of involucrin in SCC9 (Jessen *et al.*, 2001), as in normal keratinocytes (Eckert *et al.*, 2004), their mediation of oxygen effects was explored. AP1 response elements are functional in the promoters of a variety of keratinocyte differentiation marker genes, and AP1 transcription factors can participate in expression of genes such as cyclooxygenase-2 (Chun *et al.*, 2004; Chen *et al.*, 2005) as well as in wound healing more generally (Angel *et al.*, 2001).

The discovery that Jun and Fos proteins each have a sensitive cysteine residue in the DNA-binding domain that affects transcriptional activity led to the realization that AP1 transcription factors are redox-regulated (Abate *et al.*, 1990). A variety of studies have shown that expression of AP1 transcription factors is inducible by hypoxia, in some cases, accompanied by increased AP1 response element-binding activity, but demonstration of parallel expression of genes with such elements has not been straightforward (Bunn and Poyton, 1996). In human keratinocyte cultures grown in 21% oxygen, involucrin mRNA (Mazina *et al.*, 2001) and protein (Rice *et al.*, 1988) are induced strongly, but not until a week or more after confluence. EMSA supershift experiments over this period exhibited loss of Fra1- and JunB-binding activity

and appearance of c-Fos binding, a transition that did not occur in cultures maintained in 2% oxygen. The cultures maintained in 5% oxygen exhibited detectable c-Fos and a lack of Fra1 a week after confluence, approximately an intermediate state. These findings provide a plausible basis for the effect of oxygen level on differentiation marker expression and a pathway for future exploration. As measurements of mRNA and protein levels of these factors after confluence showed only minor effects of altered oxygen conditions (see Figures S1 and S2), the roles of post-translational modifications and of other AP1-interacting protein families (e.g., MAF, ATF) merit investigation.

MATERIALS AND METHODS

Cell culture

hEp from neonatal foreskin, minimally deviated SIK (passages 20–25) spontaneously immortalized human epidermal cells (Rice *et al.*, 1993), and the SCC9 human squamous carcinoma line (Rheinwald and Beckett, 1981) were serially passaged using a lethally irradiated 3T3 feeder layer in DMEM:F12 (2:1) containing 25 mM Hepes buffer, 0.18 mM adenine, 5 μ g/ml insulin, 5 μ g/ml transferrin, and 20 pM triiodothyronine (Allen-Hoffmann and Rheinwald, 1984). Normal human epidermal and SIK cultures were supplemented with EGF (10 ng/ml), except as noted. The culture incubator (ThermoForma 3110) regulated carbon dioxide at 5% and oxygen as indicated using nitrogen displacement. The human cells employed (otherwise discarded) were obtained with institutional approval.

Quantitative PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), reverse transcribed, and specific mRNAs were quantitated by real-time PCR as described previously (Patterson *et al.*, 2003). Kits for Taqman-based gene expression assays were purchased from Applied Biosystems (Foster City, CA). The mRNA levels for each target gene were calculated relative to untreated cultures and normalized (Wong and Medrano, 2005) to the following housekeeping genes: acidic ribosomal phosphoprotein P0, TATA-binding protein, and 18S RNA. Data were analyzed for triplicate cultures in duplicate experiments.

EMSA

Probes were made by labeling annealed oligonucleotides containing a single added 5'G overhang with the Klenow fragment of DNA Pol I (Promega, Madison, WI) in the presence of [³²P]dCTP (Perkin Elmer, Wellesley, MA). Binding reactions (20 μ l) contained 5–10 μ g of nuclear extract and 500 ng of poly(dI-dC)-poly(dI-dC) in a solution of 12.5 mM Hepes (pH 7.9 at 4°C), 0.2 mM EDTA, 0.01% NP-40, 0.5 mM dithiothreitol, 100 mM NaCl, and 10% glycerol. After 10 minutes incubation at room temperature, 10 fmol of labeled probe were added and incubation was continued for 20 minutes. Supershift analysis of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) family member binding was performed by adding 2 μ g of antibody (sc45x, sc46x, sc74x, sc52x, sc48x, sc605x, sc604x, respectively, from Santa Cruz Biotech, Santa Cruz, CA) to binding reactions, followed by incubation on ice for 3 hours before probe addition. DNA-protein complexes were separated on 4% polyacrylamide gels in buffer containing 25 mM Tris-HCl, 190 mM glycine, and 1 mM EDTA. The double-stranded AP1 oligonucleotide employed, corresponding to the functional AP1 site (AP1-1) in the proximal

involucrin promoter (Welter *et al.*, 1995), had the following sequence (sense strand): 5'- GCTGTGGTGAGTCAGGAAGGGGT-TAGAGGAA-3'.

Glutathione determination

Cultured keratinocytes were rinsed with EDTA (0.5 mM in isotonic saline) and supernatants from perchloric acid homogenates were submitted to reverse-phase HPLC coupled with electrochemical detection (Lakritz *et al.*, 1997). Data were normalized to cellular protein as determined by assay with bicinchoninic acid (Smith *et al.*, 1985). Reduced glutathione calculations were made against a standard curve in the range of 0.5–1,000 ng (linear relation, $R^2 = 0.996$).

Statistics

Each experiment was performed at least twice, with each sample in triplicate. Data represent mean values \pm SD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Expression of AP1 transcription factors.

Figure S2. Immunoblotting of AP1 proteins.

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