Differentiation of Cultured Human Epidermal Keratinocytes at High Cell Densities is Mediated by Endogenous Activation of the Protein Kinase C Signaling Pathway

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Normal human epidermal keratinocytes (NHEK) grown in serum-free medium on a plastic substrate spontaneously differentiate at high cell densities *in vitro*. Because protein kinase C (PKC) regulates murine keratinocyte differentiation triggered by a variety of stimuli, we examined the role of this signaling pathway in density-dependent activation of NHEK differentiation. Relative to subconfluent cultures, confluent NHEK expressed markedly higher levels of multiple differentiation markers assayed by immunoblotting, including keratin 1, loricrin, filaggrin, involucrin, TG_K, and SPR-1. Expression of several of these markers continued to increase for several days after cells reached confluency. The total level of several PKC isoforms was not substantially altered in NHEK harvested at different cell densities, based on immunoblotting; however, subcellular fractionation revealed that PKCα underwent a redistribution to the particulate fraction in confluent and postconfluent NHEK cultures, suggesting that this isozyme was activated under these conditions and may be involved in triggering the terminal differentiation program. Supporting this concept, inhibition of PKC function using bryostatin 1 or GF 109203X blocked the induction of keratinocyte differentiation markers at high cell densities. These data suggest that endogenous activation of PKC is responsible for cell density-mediated stimulation of NHEK differentiation, establishing a critical role for this pathway in regulating human as well as murine keratinocyte differentiation. ***Key words: bryostatin/filaggrin/involucrin/keratins/loricrin/SPR-1. J Invest Dermatol 111:762–766, 1998***

Normal epidermis consists of four cellular compartments that are defined by the expression of specific sets of keratinocyte differentiation markers occurring in a step-wise manner. For example, the transcripts for keratins K5 and K14 are expressed in the basal cell compartment, whereas K1 and K10 are induced in the spinous compartment; loricrin and filaggrin are first expressed in the granular compartment; and anucleate cornified cell envelopes comprise the most superficial cornified layer. The ability to induce many aspects of epidermal differentiation in cultured keratinocytes has facilitated analysis of the molecular regulation of keratinocyte-specific gene expression, as well as the role of intermediary signaling pathways in triggering this process (Yuspa, 1994).

Primary keratinocytes obtained from newborn mice can be maintained as a basal cell-like population by growing them in medium containing a reduced Ca^{2+} concentration (0.05 mM). Terminal differentiation, characterized by the sequential appearance of spinous followed by granular cell differentiation markers, is induced by raising the Ca^{2+} to 0.12 mM (Yuspa *et al*, 1989). The activation of protein kinase C (PKC) is essential for the regulation of genes involved in Ca^{2+}-induced mouse epidermal keratinocyte differentiation, because PKC inhibitors block differentiation-specific gene induction and PKC activators induce differentiation markers (Dlugosz and Yuspa, 1993, 1994). Furthermore, the subcellular distribution of PKCα, PKCδ, and PKCε is altered in differentiating keratinocytes, suggesting that they are activated during this process (Denning *et al*, 1995). *In vivo*, PKCη is selectively expressed in the granular cell compartment, and the abundance of this isoform also increases in both the membrane and the cytosol fractions in differentiating keratinocytes *in vitro* (Koizumi *et al*, 1993; Denning *et al*, 1995). In cultures of differentiating keratinocytes, both intracellular diacylglycerol, an endogenous PKC activator, and free Ca^{2+} increase, suggesting a mechanism that may lead to PKC activation (Tang *et al*, 1988; Sharpe *et al*, 1989; Kruszewski *et al*, 1991; Lee and Yuspa, 1991; Punnonen *et al*, 1993). Direct evidence that PKC regulates the expression of markers of differentiation comes from studies with a PKCα anti-sense oligonucleotide that reduces or prevents the expression of granular cell proteins in mouse keratinocytes induced to differentiate by Ca^{2+} (Lee *et al*, 1997). The α isoform of PKC was also implicated as an essential regulator of marker expression using isoform-selective pharmacologic PKC inhibitors (Denning *et al*, 1995).

The same complement of PKC isoforms is detected in both mouse and human keratinocytes (Osada *et al*, 1990; Dlugosz *et al*, 1992; Gherzi *et al*, 1992; Matsui *et al*, 1992; Fisher *et al*, 1993), suggesting that individual isoforms will have similar functions in the skin of both species. In fact, the prevailing evidence indicates that PKC is activated in differentiating human keratinocytes and regulates expression of certain suprabasal markers (Ueda *et al*, 1996; Gherzi *et al*, 1992; Matsui *et al*, 1992), but a comprehensive analysis of this relationship is lacking. Because changes in PKC have been detected in human skin diseases

Manuscript received January 19, 1998; revised April 24, 1998; accepted for publication June 26, 1998.

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Abbreviation: NHEK, normal human epidermal keratinocytes.

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(Fisher *et al*, 1993; Koizumi *et al*, 1993; Rasmussen and Celis, 1993), an understanding of the functional contribution of specific PKC isozymes to the regulation of human epidermal physiology is important for the identification of potential therapeutic targets for PKC inhibitors or activators. Furthermore, human keratinocytes in culture can be induced to differentiate by manipulating the cell density (Pillai *et al*, 1990; Hohl *et al*, 1991; Poumay and Pittelkow, 1995), and this could provide additional insights beyond the information obtained from mouse keratinocytes induced to differentiate by Ca^{2+}, because PKC α is calcium dependent and might be activated unnaturally in the mouse system. In this report we examine the role of PKC in the expression of differentiation markers in human keratinocytes obtained from adult and neonatal skin and induced to differentiate in culture by manipulating the cell density. The results reveal the crucial importance of PKC activation in marker expression and support a particularly influential contribution of PKCα.

MATERIALS AND METHODS

Reagents Bryostatin 1 was obtained from the Pharmaceutical Resources Branch of the National Cancer Institute and LC Laboratories (Woburn, MA) and GF109203X was purchased from Calbiochem (La Jolla, CA). All reagents were dissolved in dimethylsulfoxide and stored at –20°C.

Cell culture Human breast-derived adult keratinocytes and foreskin-derived neonatal keratinocytes were purchased from Clonetics (San Diego, CA), and were grown as submerged cultures on plastic dishes (Falcon Labware, Oxford, CA) in modified MCDB 153 medium (KGM, GIBCO BRL, Gaithersburg, MD) containing 0.09 mM Ca^{2+}, epidermal growth factor (5 ng per ml), and bovine pituitary extract (50 μg per ml). After the cells attained 50% confluence, the medium was changed to 0.09 mM Ca^{2+} containing KGM without epidermal growth factor and BPE.

Cell fractionation Cells were washed once with ice-cold phosphate-buffered saline and scraped into PKC lysis buffer [20 mM Tris-HCI (pH 7.5), 5 mM ethylenediamine tetraacetic acid, 0.25 mM phenylmethylsulfonylfluoride, 40 μg leupeptin per ml]. After brief sonication, the lysate was centrifuged at 100,000 × *g* for 1 h, and the supernatant was taken as the soluble fraction. The pellet was resuspended in PKC lysis buffer containing 1% Triton X-100, centrifuged as before, and the supernatant was removed for the particulate fraction. Cell fractionation was performed in the presence of the divalent cation chelator ethylenediamine tetraacetic acid to avoid the effect of elevated Ca^{2+} on membrane partitioning of PKC during the fractionation procedure (Phillips *et al*, 1989; Stabel and Parker, 1991). Protein concentration was determined by the Bio-Rad protein assay.

Western blots For total sodium dodecyl sulfate lysates, the cells were washed twice with ice-cold phosphate-buffered saline and scraped into sodium dodecyl sulfate sample buffer (Laemmli, 1970), boiled, and run immediately on polyacrylamide gels. For soluble and particulate PKC lysates, 30 μg of each protein fraction was loaded per lane. Proteins were transferred to nitrocellulose electrophoretically, and the membranes were blocked in 5% milk in tris-buffered saline. For detection of PKC isozymes, the membranes were incubated with anti-PKC antibodies specific for the catalytic subunit of PKCα at a dilution of 1:500 (Upstate Biotechnology, Lake Placid, NY), PKCδ and PKCη at a 1:100 dilution (Santa Cruz Biotechnology, Santa Cruz, CA), and PKCε at a dilution of 1:1000 (GIBCO BRL, Gaithersburg, MD). Human filaggrin and involucrin were used at 1:200 (Biomedical Technology, Stoughton MA), c-terminal human transglutaminase K (TG_K) antibody was a gift from Dr. Robert Rice (University of California, Davis, CA), and K1, K14, loricrin, and SPR-1 were detected with antibodies described previously (Roop *et al*, 1985; Denning *et al*, 1995; Kartasova *et al*, 1996). Proteins were detected using the ECL system (Amersham, Arlington Heights, IL) with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) at a 1:5000 dilution. All PKC antibodies were isozyme specific as determined by the lack of cross-reactivity to purified recombinant PKC isozymes isolated from a baculovirus expression system.

RESULTS

Increasing cell density upregulates expression of multiple differentiation markers in cultured human keratinocytes The expression of markers characteristic of spinous and granular cell differentiation was evaluated in low-passage adult and neonatal normal human epidermal keratinocytes (NHEK) cultures at three cell densities: preconfluent (≈70%–80% confluent), confluent, and postconfluent (three additional days after reaching confluence). Confluency was

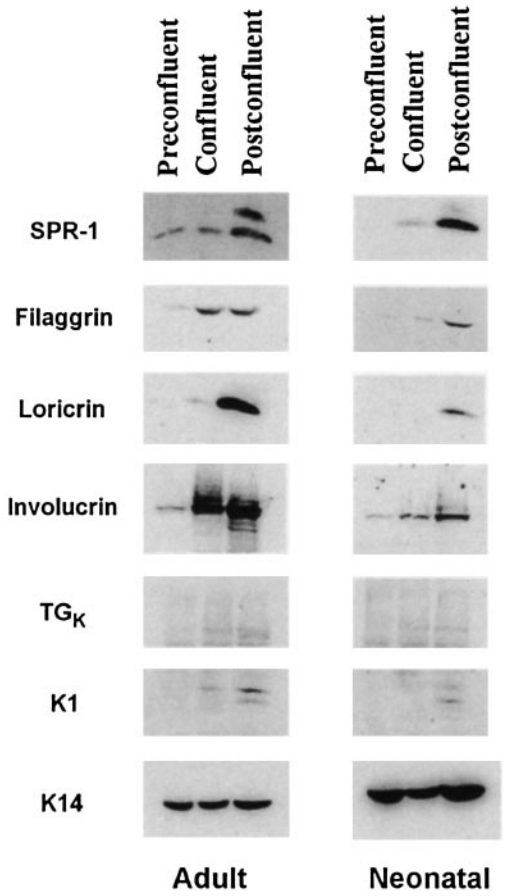

Figure 1. Cell density induces expression of multiple keratinocyte-specific differentiation markers in cultured NHEK. Keratinocytes isolated from either adult or neonatal human skin were analyzed when 70%–80% confluent, 100% confluent, and 3 d postconfluent. Polyacrylamide gel electrophoresis, transfer to nitrocellulose, and immunoblotting were performed using total cells lysates as described in *Materials and Methods*. Note density-dependent upregulation of spinous and granular cell differentiation markers, with little effect on the abundance of the basal cell keratin K14. The nature of the higher molecular weight band in differentiating keratinocytes detected using SPR-1 antibody is unknown.

asssociated with increased expression of all the granular cell markers examined in both neonatal and adult cells (**Fig 1**) and in most cases, expression increased further in the postconfluent state (note SPR-1, filaggrin, loricrin, and involucrin). Confluency in this study appeared to influence the spinous marker K1, as reported previously (Poumay and Pittelkow, 1995; Pillai *et al*, 1990), whereas K14, a basal cell keratin, was not altered by confluency (**Fig 1**). Although similar results were obtained with both adult and neonatal cells, marker expression generally occurred earlier in confluency in adult keratinocyte cultures than in neonatal cells.

Increasing cell density is associated with selective translocation of PKCα to the particulate fraction in cultured human keratinocytes Additional studies were performed to determine whether confluency-mediated keratinocyte differentiation was associated with alterations in the abundance or distribution of specific PKC isoforms. Major changes in the total levels of PKCδ, PKCε, or PKCη were not observed when comparing preconfluent, confluent, and postconfluent keratinocyte cultures, although a modest increase in PKCα levels was detected (**Fig 2**). More significantly, fractionation of keratinocytes revealed the appearance of PKCα in particulate fractions of confluent and postconfluent, but not subconfluent cultures (**Fig 3**). Because activation of PKCα is associated with translocation from the cytosol to plasma membrane, these findings suggest that density-mediated keratinocyte differentiation is associated with endogenous

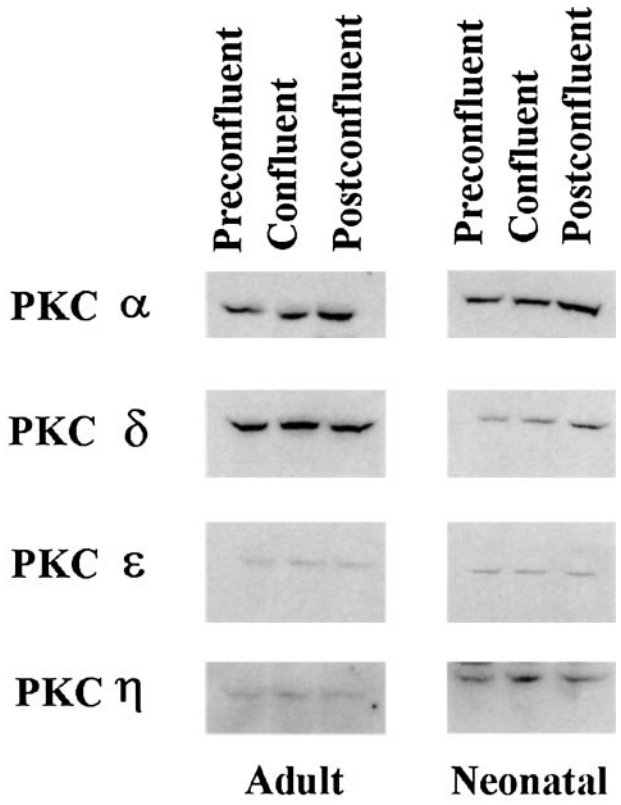

Figure 2. Increased cell density is not associated with major changes in the abundance of PKC isoforms in cultured NHEK. Immunoblot analysis was performed using Triton X-100 lysates prepared from newborn and adult NHEK at different stages of confluency. The expression level of PKC isozymes was determined using isozyme-specific antibodies as described in *Materials and Methods*.

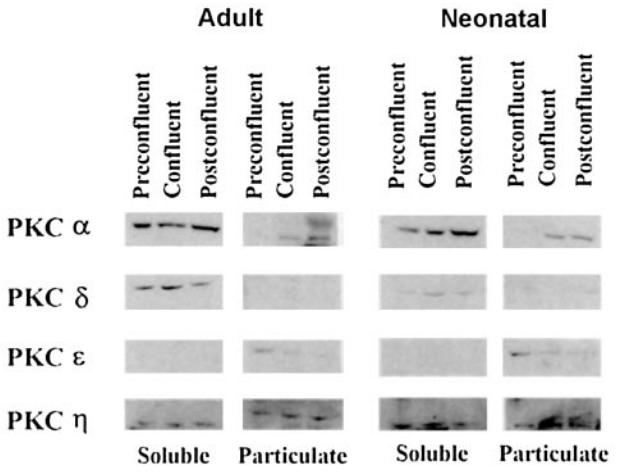

Figure 3. PKCα accumulates in the particulate fraction of NHEK growing at increased cell densities. Soluble and particulate protein fractions were prepared from keratinocytes at different cell densities as described in *Materials and Methods*. The expression level of PKC isozymes in these two compartments was assessed by immunoblotting.

activation of this PKC isoform. The modest level of PKCα detected in the particulate fraction is entirely consistent with a physiologic activation event, illustrated by the modest translocation of PKCα also detected in mouse epidermal keratinocytes induced to terminally differentiate with Ca^{2+} (Denning *et al*, 1995). Of the other isoforms examined, PKCδ was primarily cytosolic whereas PKCε was primarily particulate (**Fig 3**). Interestingly, there was a reduction in the abundance

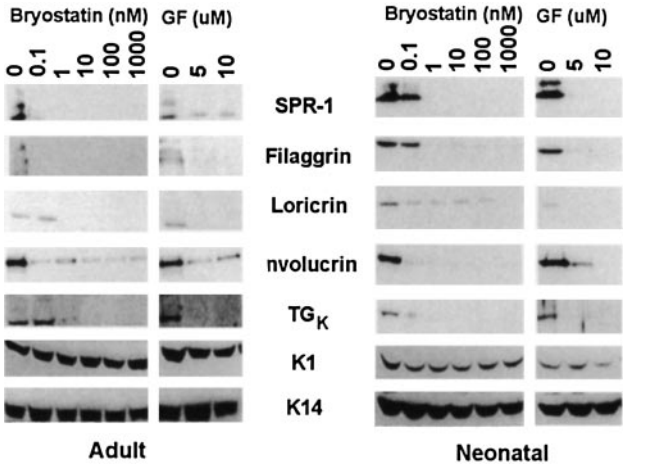

Figure 4. Density-mediated expression of keratinocyte differentiation markers is blocked by inhibiting the PKC pathway. Treatment with the indicated concentrations of bryostatin 1 or GF 109203X (GF) was initiated when cultures were preconfluent. Cells were grown for 96 h, until postconfluent, in the presence of inhibitors. Total cell lysates were prepared and immunoblotting performed as in **Fig 1**. Note the selective inhibition of all granular cell differentiation markers examined, but not keratins K1 and K14.

of particulate PKCε in confluent and postconfluent cultures (**Fig 3**). PKCη remained evenly distributed among each compartment in the differing states of confluency. It is unlikely that the appearance of PKCα in the particulate fraction of differentiating keratinocytes was an artifact related to protein cross-linking, as neither PKCδ nor PKCη exhibited convincing evidence of retention in this cellular fraction (**Fig 3**).

Inhibition of PKC blocks cell density-mediated keratinocyte differentiation The foregoing studies demonstrated a temporal correlation between induction of human keratinocyte differentiation by confluency and the activation of PKCα. To provide more direct evidence for the causal role of PKC activation in the differentiation response, specific inhibitors of PKC that work by different mechanisms were employed. These had previously been used to evaluate PKC-mediated events in mouse keratinocyte cultures. Bryostatin 1 is a natural product with a high affinity for binding to PKC (Pettit, 1991), that transiently activates PKC but subsequently causes downregulation in an isoform-specific manner (Szallasi *et al*, 1994; Denning *et al*, 1995). Bryostatin 1 inhibited expression of granular cell markers in both adult and neonatal confluent keratinocytes at doses of 0.1–1 nM, but did not inhibit expression of K1 or K14, suggesting that PKC is less important in cell density-mediated regulation of basal or spinous cell markers (**Fig 4**). Another specific inhibitor of PKC is GF 109203X, which competes at the ATP binding site (Toullec *et al*, 1991). GF 109203X also effectively inhibits granular cell differentiation marker expression at both 1 and 5 μM concentrations (**Fig 4**). Like bryostatin 1, GF109203X did not alter expression of K1 or K14. Neither PKC inhibitor was toxic to keratinocytes, because total cell number was not reduced in treated cultures when compared with untreated controls after a 96 h exposure (data not shown). These data also indicate that the inhibitory effect of PKC inhibitors on keratinocyte differentiation was not due to growth suppression and a resultant reduction in cell density.

DISCUSSION

The results reported here suggest that PKC activation is required for the expression of granular cell differentiation markers in cultured human keratinocytes, independent of changes in extracellular Ca^{2+}. Previous studies had shown that both PKC activity (Gherzi *et al*, 1992; Matsui *et al*, 1992) and ligand binding (Snoek *et al*, 1987) increased in human keratinocytes induced to differentiate by raising extracellular Ca^{2+}. Because the spinous cell marker K1 does not require PKC

activation for expression, we conclude that PKC regulates the spinous to granular transition in human keratinocytes induced to differentiate by confluency, as we have shown previously for mouse keratinocytes induced by Ca^{2+} (Dlugosz and Yuspa, 1993, 1994). Although our study only examined marker expression at the protein level, it is likely that PKC is regulating differentiation by influencing gene expression, because prior studies designed to examine this relationship indicate that differentiation markers are controlled at the transcriptional level (Dlugosz and Yuspa, 1993). Of relevance to this issue is a previous study demonstrating upregulation of loricrin mRNA expression in postconfluent human keratinocyte cultures (Hohl *et al*, 1991). This report also confirmed that the changes in loricrin gene expression were due to confluency status, rather than the amount of time during which the cells were kept in culture.

One downstream target for PKC action could be the AP-1 transcription factor family that is known to be a target for PKC in keratinocytes and is a component of the regulatory region of genes for the granular cell markers that we have studied (Rutberg *et al*, 1996). Other studies have employed exogenous activators of PKC and cultured human keratinocytes to demonstrate a role for PKC in expression of collagenase (Sudbeck *et al*, 1994), cytochrome p-4501 A1 (Berghard *et al*, 1993), ornithine decarboxylase (Verma *et al*, 1985), and involucrin (Jones and Sharpe, 1994), and a selective role for PKCη in the induction of transglutaminase gene expression (Ueda *et al*, 1996).

Although the activation of PKC during keratinocyte differentiation has been demonstrated in several reports, we observed only minor changes in the total levels of PKC isozymes, although the redistribution of PKCα is taken as an indication of this isozyme's activation. This differs from several previous reports in which differentiation was induced by Ca^{2+} or stratification and increases in PKC mRNA or protein were detected (Gherzi *et al*, 1992; Matsui *et al*, 1992), suggesting that confluency may involve a change in intracellular activators of PKC. Both diacylglycerol and cholesterol sulfate are known to increase in differentiating human keratinocytes (Jetten *et al*, 1989), and confluency is associated with a rise in intracellular Ca^{2+} (Pillai *et al*, 1991). Furthermore, exogenous phospholipase C can mimic the effects of phorbol esters on cultured human keratinocytes and induce cornification (Parkinson, 1987), confirming that upregulation of diacylglycerol can activate PKC in keratinocytes.

The elucidation of the signaling pathways that regulate epidermal differentiation is important for understanding the pathophysiology of skin disease and for designing pharmaceuticals for rational therapies. In particular, the PKC pathway appears to be impacted in psoriasis, where the enzyme activity levels and distribution are altered, presumably as a consequence of overactivity of phospholipase C and elevation of diacylglycerol (Horn *et al*, 1987; Fisher *et al*, 1990, 1993; Koizumi *et al*, 1993; Rasmussen and Celis, 1993). Such a change might be expected to alter the onset of terminal differentiation, and this is consistent with the pathology of that disease. Although the precise isoforms involved in this change are not clearly defined and some overlap in function may be expected, advances in drug design or molecular approaches to cutaneous therapies may provide clinicians with specific drugs to inhibit PKC isoforms and to modify signaling in this pathway.

We are grateful to Drs. Robert Rice and Tonya Kartasova for generously providing antibodies, and appreciate the secretarial assistance of Margaret Taylor.

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