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population to which the results will later be applied. In the present study, neither physicians nor patients were randomly selected. The convenience sample of 15 volunteer patients, whose characteristics (age, sex, subtypes of pemphigus, time in the course of the disease, i.e., before or after treatment) are not described, cannot be assumed to represent patients who may be included in future trials. The PV-to-PF ratio was not provided—PV accounts for about two-thirds of all pemphigus cases and PF for about 20%. These points are important, because the clinical characteristics included in the scale (mucous membrane involvement), prognosis, and therapeutics differ between PV and PF, and it is impossible to estimate to which population the results could be extrapolated.

Overall, validated outcome measures are essential for optimal patient care. Therefore, the successive research work of this International Pemphigus Committee is of major importance, and this study on outcome instruments for pemphigus is a first approach. The assessment of larger, well-defined populations with a formal evaluation, including the usual successive methodological steps, is needed before the PDAI should be used as a research instrument.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Protein Kinase Cα Puts the Handcuffs on Epidermal Keratinocyte Proliferation

Wendy B. Bollag[1,2]

As the predominant cellular receptor for phorbol esters, protein kinase C (PKC) is assumed to play a role in epidermal carcinogenesis. Nevertheless, determining its exact role in keratinocytes has been difficult because of the existence of multiple PKC isoforms and the inherent weaknesses in methodologies used to investigate their function. In this issue, Jerome-Morais *et al.* describe their use of multiple *in vitro, in situ*, overexpression, and knockdown approaches to demonstrate that PKCα induces keratinocyte growth arrest.

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The ability of phorbol esters to promote tumor formation in mouse epidermis preinitiated with a carcinogen led to the idea that the phorbol ester target in skin must be involved in epidermal tumorigenesis. Thus, the identification of protein kinase C (PKC) as the primary cellular target of phorbol esters suggested an important role for PKC in epidermal tumor formation, as well as for the biology of the keratinocyte, the predominant cell in the epidermis (reviewed in Bollag and Bollag, 2001). However, despite an abundance of studies investigating the role of this enzyme in the epidermis, both *in vitro* and *in vivo*, the exact role of PKC in keratinocyte function and epidermal tumorigenesis remains unclear.

This imperfect knowledge about the role played by PKC in the epidermis is likely due in large part to the existence of multiple PKC family members, each with disparate mechanisms of activation and different function (reviewed in Bollag and Bollag, 2001). Thus, conventional PKC isoenzymes (PKCα, -βI, -βII, and -γ) are phospholipid dependent, diacylglycerol activated, and calcium sensitive, whereas the novel PKC isoforms (PKCδ, -ε, -η, and -θ) also require phospholipids and are activated by diacylglycerol, but they are insensitive to calcium. Atypical PKCs (PKCζ and -λ/ι), on the other hand, require neither diacylglycerol nor calcium (Figure 1). Because phorbol esters can substitute for naturally occurring diacylglycerol in activating conventional and novel PKC isoenzymes, the ability of phorbol esters to promote epidermal tumor formation indicates the

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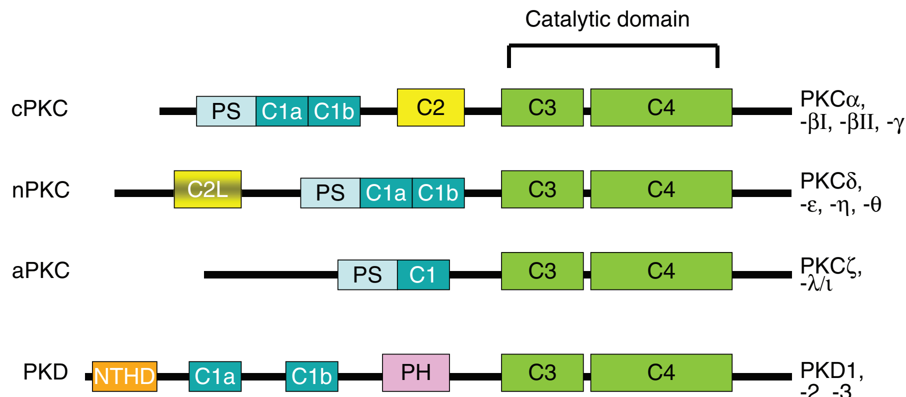

Figure 1. Protein kinase C isoenzyme structure. PKC isoforms can be divided into three subtypes: the conventional, or classic, PKCs (cPKCs); the novel PKCs (nPKCs); and the atypical PKCs (aPKCs). The cPKC and nPKC isoenzymes possess the following domains: the pseudosubstrate (PS) domain, which in the unstimulated state interacts with the substrate binding domain and inhibits PKC activity; the two C1 domains that bind diacylglycerol or phorbol ester; the C2 domain that binds calcium (a C2-like (C2L) domain found in nPKCs does not bind calcium but mediates protein–protein interactions); and the catalytic domain of the enzyme (formed by C3 and C4). On the other hand, aPKCs possess only one C1 domain and thus do not bind diacylglycerol; likewise, they lack a C2 domain. Protein kinase D (PKD), originally described as PKCμ but now placed in a separate kinase family, possesses two C1 domains, allowing diacylglycerol/phorbol ester binding, as well as the catalytic domain, which has greater homology to calcium/calmodulin-dependent protein kinases than to PKCs. In addition, PKD possesses two domains not observed in PKCs: an N-terminal hydrophobic domain (NTHD) of unknown function and a pleckstrin homology (PH) domain involved in protein–protein interactions.

importance of these targets in keratinocyte biology. However, because phorbol esters can activate all eight classic and novel PKC isoforms, the specific isoform(s) involved in tumor promotion remains uncertain.

Another difficulty in achieving a complete understanding of the role of PKC isoforms in epidermal tumorigenesis is related to the relative nonspecificity of the phorbol ester tumor promoters themselves. Thus, in addition to stimulating the activity of all of the conventional and novel PKC isoenzymes, phorbol esters can also activate some non-PKC targets, including Ras-GRP1 (a guanine nucleotide exchange factor for Ras), chimaerins (Rac GTPase-activating proteins), Munc13s (involved in synaptic vesicle priming), diacylglycerol kinase-γ (a diacylglycerol-metabolizing enzyme), and protein kinase D (PKD; reviewed in Brose and Rosenmund, 2002). (Although PKD has also been described as PKCμ, this enzyme and related PKD2 and 3 are now classified as members of a kinase family separate from the PKCs (Brose and Rosenmund, 2002).) In addition, phorbol esters can induce not only the activation of PKC but also the proteolytic degradation (downregulation) of many of the isoforms. Therefore, it is difficult to determine which of phorbol esters' PKC or non-PKC targets underlies tumor promotion and whether the response is the result of activation or downregulation of a particular (or several) PKC isozyme(s).

Our knowledge about the role of the PKC isoforms in the epidermis is also complicated by the various approaches, with their attendant advantages and disadvantages, used to study their function. Many of the PKC isoenzymes have been studied *in vitro*, using both overexpression and knockdown; similarly, genetically engineered mice both overexpressing and lacking the genes for some of the isoforms have been generated. Overexpression studies have the potential difficulty that the overexpressed protein, *in vitro* or *in vivo*, may interact with proteins or substrates and/or localize to regions of the cells not usually accessed by the endogenous protein. On the other hand, most knockdown strategies have the potential for artifacts, and knockout mice can have alterations in other pathways to compensate for the loss of the deleted gene during development. In addition, possible cell autonomous versus cell microenvironmental effects may exist *in vivo* (see below). All of these issues may complicate interpretation of the role of a particular PKC isoenzyme in epidermal function and tumorigenesis and necessitate careful studies using multiple approaches, such as the work reported by Jerome-Morais *et al*. in this issue.

Of the conventional PKC isoforms expressed in the epidermis, PKCα is the most abundant. (Although some investigators have reported that PKCα is the only conventional isoenzyme found in epidermal keratinocytes, recent data suggest that PKCβII may also be expressed in keratinocytes (our unpublished results and Hara *et al.,* 2005).) *In vitro*, adenovirus-mediated overexpression of the PKCα isoform, as well as PKCδ, -ε, and -η, induces the expression of involucrin (reviewed in Bollag and Bollag, 2001), as well as keratins 1 and 10, loricrin, and filaggrin (Seo *et al*., 2004), although there is no effect on growth arrest or transglutaminase activity (Ohba *et al*., 1998). Furthermore, overexpression of a dominant negative mutant or antisense ablation of PKCα inhibits the expression of multiple differentiation markers in response to elevated extracellular calcium levels, a known keratinocyte-differentiating agent (Seo *et al*., 2004, and reviewed in Bollag and Bollag, 2001). Together, these results indicate a role for PKCα in promoting keratinocyte differentiation.

This interpretation has been largely confirmed, although it is somewhat disputed by data obtained *in vivo*. In transgenic mice engineered to overexpress PKCα in the skin, no effect on carcinogen-initiated, phorbol ester–promoted tumorigenesis is observed despite a striking effect of this overexpression on inflammation in the skin (Cataisson *et al*., 2005; Jansen *et al*., 2001; Wang *et al*., 1999). On the other hand, in PKCα knockout mice Hara *et al.* (2005) observed an impairment of epidermal hyperplasia in response to tumor promoter application, but an increased sensitivity to tumor formation. Changes in tumor formation, however, may not be related only to effects in keratinocytes. It is thought that cell types in addition to keratinocytes are involved in epidermal tumorigenesis; therefore, total ablation of a gene in all cell types can complicate interpretation of the results obtained because it becomes unclear whether any

alteration in tumor formation is caused by a lack of the gene/protein in the epidermal keratinocytes themselves (i.e., cell autonomous) or its absence in cells that communicate with the keratinocytes during tumorigenesis (e.g., in dermal fibroblasts and infiltrating immune cells and their ability to generate a particular microenvironment).

> Multi-dimensional cultures and overexpression and knockdown methodologies are required to study epidermal signaling.

As an example, in null mice lacking phospholipase Cε, a resistance to tumorigenesis in the two-stage chemical carcinogenesis protocol is observed; however, this resistance is the result of alterations not in the keratinocytes themselves but in the inflammatory response initiated by phorbol esters (Ikuta *et al.*, 2008). Thus, total ablation of PKCα may result in a different phenotype than might be observed in mice in which PKCα loss is restricted to epidermal keratinocytes. Furthermore, although mice have the advantage of being genetically tractable with skin that is a reasonable model for the human organ, there are some differences in the epidermis of the two species, necessitating research strategies that are amenable to human keratinocytes as well in order to address these potential disparities. Thus, through a variety of approaches *in vitro* and *in vivo*, clues have been obtained concerning the role of PKCα in epidermal keratinocyte biology. Nevertheless, additional studies are needed to further define the important function(s) of this enzyme.

In this issue, Jerome-Morais *et al.* use multiple approaches to dissect the role of PKCα in keratinocytes. First, the authors were able to demonstrate in human epidermis *in situ* that PKCα is localized in the plasma membrane of cells of the first spinous layer. Because PKCα requires phospholipid for activation, membrane-located protein kinase is thought to represent activated enzyme. However, and perhaps more important, they also used a carefully validated antibody that detects PKCα-mediated phosphorylation of PKCα substrates on serine and threonine residues to demonstrate that this enzyme was indeed active in the spinous layer. In addition, using overexpression of a constitutively active PKCα, Jerome-Morais *et al.* (2009) showed that PKC activity results in keratinocyte growth arrest, as monitored by decreases in cell number, colony-forming efficiency, and radiolabeled thymidine incorporation into DNA (a measure of DNA synthesis) and an increase in the percentage of cells in the G0/G1 phase of the cell cycle (with a concomitant reduction of the percentage in the S phase). These authors then utilized the converse approach, PKCα knockdown with RNA interference technology, as well as a three-dimensional organotypic culture system, to demonstrate that decreases in PKCα levels resulted in a thinner organotypic raft epidermis despite an increase in proliferation in the culture. This apparently paradoxical result is nevertheless consistent with the findings of Hara *et al.* (2005), in which mice lacking PKCα exhibited reduced epidermal thickness after wound healing but enhanced tumorigenesis upon carcinogen initiation and tumor promotion. Together, the results suggest multiple roles of PKCα in epidermal keratinocytes: not only as a tumor-suppressive, growth-inhibitory signal but also as a migration- and/or stratification-inducing signal, as discussed in the article by Jerome-Morais *et al.* It should be noted, however, that not all of the cellular functions of PKCα may require catalytic activity. As an example, kinase-dead PKCα can activate phospholipase D (e.g., Oka *et al.*, 2003); it will therefore be of interest to ascertain which of the defects observed with PKCα knockdown can be rescued by wild-type versus catalytically inactive mutant PKCα.

The complex regulation of the epidermal keratinocyte program of self-renewal, proliferation, and differentiation makes it difficult to decipher the signaling pathways mediating these processes. However, because of the likely contributions of alterations in these pathways to the etiology of skin diseases, including tumorigenesis, studies to decode the secrets of these cells are imperative. As exemplified in the report by Jerome-Morais *et al.*, the use of two- and three-dimensional human keratinocyte cultures, as well as both overexpression and knockdown methodologies, represents a sound and multifaceted approach to investigating the complicated signaling pathways regulating epidermal structure and function.

CONFLICT OF INTEREST

The author states no conflict of interest.

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