THESIS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY (Ph.D.)

ROLES OF PROTEIN KINASE C ISOENZYMES IN REGULATION OF CELLULAR FUNCTIONS OF HUMAN HaCaT KERATINOCYTES

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INTRODUCTION

Signal transduction, proliferation, differentiation

The regulation of cellular mechanisms occurs via various signal transduction processes. These events involve the binding of the agonist with its receptor, activation of transmembrane proteins and enzymes, production of secondary messengers, and activation of intracellular enzymes. Among these molecules, the function of protein kinases – by incorporating the terminal phosphate group of (mostly the) ATP to the hydroxyl residues of various side-chains of proteins – is to catalyze the reversible phosphorylation of most of the cellular proteins. These phosphorylation processes play a central role in the regulation of the cell cycle, proliferation, and differentiation; in morphology, contractility and migration of cells; and in functions of channels, transporters, and receptor proteins.

The proliferation and differentiation are key steps in the development, growth, and physiological and injury-induced regeneration of the cells and tissues. These rather complex mechanisms are chiefly regulated, among others, by different protein kinase systems and processes modulating the intracellular calcium homeostasis. It was also shown that pathological alterations in the phosphorylation levels of certain cellular proteins and/or abnormal intracellular ionic concentrations could lead to profound modifications in the proliferation and differentiation of the given cell which, in turn, might result in such severe conditions as malignant transformation. The dramatically raising incidence and, most importantly, mortality of tumor-related human diseases, therefore, urged the experimental efforts to clarify regulatory effector mechanisms that are involved in intracellular signal transduction pathways.

The keratinocytes

In the investigations of processes of proliferation, differentiation, apoptosis, and tumor genesis and progression, one of the most often studied cell model is the keratinocytes, whose *in vivo* function is the establishment and maintenance of the outermost layer of the skin, the epidermis. These cells form a major physical-chemical barrier between the environment and the body interior to protect the organism against harmful factors. The structure and the function of the epidermis are maintained by a well-defined and -balanced program of proliferation and differentiation of the keratinocytes, establishing five layers of the epidermis. It was proven that alterations in the regulation of proliferation and of the state of differentiation result in hyperproliferative transformation and, eventually, tumor development.

Proliferation and differentiation of keratinocytes, along with their immunological and secretory functions are strongly affected by various cytokines and growth factors produced by the numerous surrounding cell types of the skin. Since the keratinocytes do express the receptors and signaling pathways specific for such agents, these cells can be regarded as ideal targets for investigating the above processes.

The HaCaT keratinocytes

One of the major problems arises in experimental dermatology is that the normal human epidermal keratinocytes (NHEK) cannot be maintained and subcultivated for a long time in culture due to the physiological induction of apoptosis. Therefore, previous studies aimed to establish such cell lines which *in vitro* well model cellular processes of NHEK yet can be kept in culture for a much longer time period. One of the most suitable cell types is the human immortalized HaCaT keratinocyte cell line. The cells are immortal but yet non-tumorigenic, their *in vitro* proliferation and *in vivo* tumorigenic potencies are significantly increased, and, although they do express various differentiation

markers similarly to those of NHEK, they lack the very final stages of terminal differentiation. Therefore, these cells are referenced as one of the best model cell line for investigating such NHEK-specific phenomena as proliferation, immortalization, transformation, and tumor progression

The protein kinase C isoenzymes

Protein kinase C (PKC) comprises a family of serine/threonine kinases. Up to date, at least 11 different PKC isoenzymes have been identified, which – based on their structural characteristics and activation mechanisms – can be classified into four groups. The "conventional" group (cPKC) contains 4 isoforms: PKCα, β_I, β_{II}, and γ. Common features of these molecules are that their activation is fully dependent of the joint presence of calcium and diacylglycerol (DAG) (or its exogenous analogues, the phorbol esters). The second group is called the "novel" PKCs (nPKC) and involves the calcium-independent PKCδ, ε, η, and θ which can be fully activated by DAG or phorbol esters. The third is the group of "atypical" (PKCζ, and λ/ι; aPKCs) isoforms which do not require calcium or DAG/phorbol ester for the activation. Finally, a separate group is formed by the unique PKCμ which bears irregular structural and activation characteristics.

Every single cell type in the human body possesses PKC isoforms! It is noteworthy, however, that not all PKC isoenzymes can be found in every cell type; i.e., PKC isoforms possess a characteristic species-, tissue-, and cell-specific expression pattern. Due to the variety of such patterns, PKC isoforms play a central role in the regulation of a wide array of physiological mechanisms such as cellular proliferation and differentiation; the processes of programmed cell death (apoptosis); the production and secretion of vasoactive substances, growth factor, and cytokines; the establishment and optimal maintenance of electrophysiological properties (channel activity, action potential coding, muscle

contractility) of excitable cell types; in the proper integrity of central and peripheral nervous systems; and in the immunological mechanisms (phagocytosis, production immunoglobulin).

Very recently it was also shown that the PKC isoforms not only possess structural, activation, and distribution heterogeneity, but their regulation and, most importantly, their isoform-specific roles are very often different from one another. It was also proven that not only may some PKC isoforms be active whereas others not for a given response (especially in the cases of regulation of proliferation and differentiation) but different PKC isozymes may have antagonistic effects on the same cellular event.

The PKC isoforms and the keratinocytes

The regulatory roles of the PKC isoforms were also described on cellular processes of keratinocytes. The studies, mostly performed on murine keratinocytes, revealed that several isoenzymes (PKCα, δ, ε, η, ζ) play regulatory roles and that chiefly the cPKCα regulates the proliferation and differentiation programs of the cells.

We possess much information about the PKC system in NHEK; although the presence of the above isoforms (PKCα, δ, ε, η, ζ) were also documented, the existence of other PKCs (PKCβ, μ) also cannot be ruled out. It was shown, however, that the expression level and the subcellular localization of several isoforms altered in parallel with the differentiation program initiated by the high extracellular concentration ($[Ca^{2+}]_e$) and/or by the high cell density (confluence).

Both on human and murine keratinocytes, the effect of PKC activator phorbol esters were also examined. These reports revealed that, on both cell types, the phorbol ester treatment (using e.g., phorbol 12-myristate 13-acetate, PMA) inhibited cellular proliferation and induced terminal differentiation of the cells. It was also shown that certain PKC inhibitors, besides stimulating

proliferation, significantly altered the expression profile of various differentiation markers indicating that the endogenous PKC system indeed plays a key role in the regulation of the above mechanisms.

Very recently, similarly to findings on murine keratinocytes, numerous reports provided evidence about the isoform-specific roles of certain PKCs on NHEK as well (PKCα was, for example, shown to regulate the onset and extension of the terminal differentiation program of the cells). Using adenoviral vectors, it was also documented that the overexpression of PKCδ or η isoenzymes, similarly to the functions of PKCα, suppressed cellular proliferation but promoted differentiation. Finally, it was also claimed that the transgenic presence of these isoforms in genetically engineered mice, unlike the overexpression of PKCε, significantly inhibited the *in vivo* growth of skin tumors.

AIMS OF THE STUDY

In contrast to murine and human keratinocytes, we possessed minor data about the regulatory roles of the PKC isoenzymes in the cellular functions of human HaCaT keratinocytes. In the very few available studies it was documented that the inhibition of the PKC system altered the morphology of the cells and the expression of certain differentiation markers. These findings, however, did not provide evidence about the direct role of the members of the PKC system in cellular events of HaCaT keratinocytes and, furthermore, they fully lacked the description of isoenzyme-specific regulatory functions.

Therefore, in our experiments presented in the Thesis, the following scientific aims were investigated:

1. Our first goal was to describe the PKC isoform pattern of the HaCaT keratinocytes. We, moreover, investigated the possible alterations in the PKC expression levels during cellular proliferation and the high cell density-mediated differentiation.
2. In addition, we examined the effect of a general PKC activator PMA on function of HaCaT keratinocytes. We intended to identify those PKC isoforms that might mediate the cellular action of PMA.
3. To clarify the specific roles of individual PKC isoforms, we employed selective and specific PKC inhibitors and measured the effect of these agents on proliferation and differentiation.
4. Using recombinant molecular biological techniques, we established HaCaT keratinocytes stably overexpressing certain PKC isoforms. On these cells, we analyzed the effect of such modifications on *in vitro* proliferation, differentiation, and apoptosis, and on morphology of the cells.
5. Finally, in our *in vivo* experiments, the PKC overexpresser HaCaT keratinocytes were injected to immuno-deficient (Severe Combined Immuno-Deficiency, SCID) mice, and, by analyzing the developed tumors, the specific roles of the PKC isoenzymes in the tumorigenetic processes were studied.

MATERIALS AND METHODS

Cell culturing

The human immortalized HaCaT keratinocyte cell line was cultured in DMEM, supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics, at 37 °C and 5 % CO_2 atmosphere.

Generation of PKC constructs

PKC constructs were engineered as described previously; the cDNA sequences of PKCα, β, δ, and ε were subcloned into a metallothionein promoter-driven eukaryotic expression vector (MTH). The vector sequence encodes a C-terminal PKCε-derived 12 amino acid tag (εMTH) and attaches it to the end of each recombinant PKC isoenzyme.

Generation of HaCaT keratinocytes overexpressing certain PKC isoforms

HaCaT cells were transfected by either the empty pεMTH vector (control cells) or by the vectors encoding the cDNA sequences of PKCα, β, δ, or ε using a Lipofectamine anionic detergent. Cells were selected in DMEM containing 750 μg/ml G418 (Geneticin), single colonies (clones) were then isolated, and the level of stable transfection and overexpression was verified by Western blotting.

Determination of viable cell numbers

The number of viable cells was determined by a colorimetric MTT assay. This procedure is based on that the mitochondrial dehydrogenase enzyme of living cells converts the tetrasolium ring of MTT (3-[4,5-dimethylthiasole-2-il]-2,5-diphyenyl tetrasolium-bromide) to formazan, the amount of which, well correlating with number of viable cells, can be determined colorimetrically.

Determination of cellular proliferation

Cellular proliferation, on the one hand, was measured by a colorimetric bromo-deoxyuridine (BrDU) assay. The basis of the method is that the incorporation of the thymidine-analogue BrDU to the DNA of proliferating cells can be colorimetrically measured using a peroxidase-conjugated anti-BrDU antibody and tetramethylbenzidine substrate.

In another experiments, to assess doubling times and maximal cell numbers of PKC overexpressers, keratinocytes in triplicate were harvested by trypsinization and counted using a hemocytometer. The following equation was used to calculate doubling time: $\tau = D/\log_2(N/N_0)$ where τ is the doubling time, D is the number of days of culturing, N and N_0 are the number of cells at the end and the beginning of the experiments, respectively. To determine the maximal cell density, cells were grown to confluence and kept post-confluent for 3 additional days and then counted as described above.

Western (immuno)blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS), harvested in homogenization buffer, and the protein content was determined. Total cell lysates were then subjected to SDS-PAGE (7.5 % gels were loaded with 20-30 μg protein per lane) and transferred to nitrocellulose membranes. Membranes were then blocked with 5% dry milk in PBS and probed with the appropriate primary antibodies against the given PKC isoforms or differentiation markers. Peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies were used as secondary antibodies, and the immunoreactive bands were visualized by an ECL Western blotting detection kit on light sensitive films.

Immunocytochemistry

Cultured HaCaT cells were fixed in ice-cold acetone and blocked in blocking solution containing bovine serum albumin. Cells were then first incubated with rabbit anti-PKC antibodies, then with a biotin-conjugated goat anti-rabbit IgG, and finally with Streptavidin-conjugated Texas Red.

Intracellular calcium imaging

A calcium sensitive probe was introduced into the intracellular space by incubating the keratinocytes with 5 μM fura-2 AM and then the changes in intracellular calcium concentration ($[Ca^{2+})_i]$) were investigated. The coverslips, containing the fura-2 loaded cells, were placed on the stage of an inverted fluorescence microscope. Excitation was altered between 340 and 380 nm using a dual wavelength monochromator, whereas the emission was monitored at 510 nm with a photomultiplier at an acquisition rate of 10 Hz per ratio. Resting $[Ca^{2+}]_i$ levels were calculated according to the method of Grynkiewicz et al. (1985). The resting $[Ca^{2+}]_i$ levels of usually 20 cells/coverslip were determined, and the values were then averaged and expressed as mean ± SEM. Statistical analysis was performed using a Student's *t*-test.

PKC activity (kinase) assay

The PKC activity of transfected HaCaT cells was determined as described before. Briefly, cells were lysed in lysis buffer, and the kinase activities of cell lysates were examined by measuring the incorporation of $[\gamma\text{-}^{32}P]$ATP using Histone III (H-III) or myosin light chain kinase 20 (MLCK20) as substrates.

Determination of apoptosis

Stable transfectants of HaCaT cells overexpressing the different PKC isoforms or the control empty vector were treated with 10 μM 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) or 50 nM tumor necrosis factor α (TNFα) for 2 days. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated annexin V and the percentage of apoptotic cells, as a function of total cell number, was determined using flow cytometry.

Investigation of tumorigenicity in SCID mice

Cells overexpressing the various PKC isoforms (along with the control HaCaT cells), at a density of 1-2 x 10^6 viable cells/200 μl, were injected intradermally to Severe Combined Immuno Deficiency (SCID) mice. Animals were euthanized after 30 days and the averaged three-dimensional size and histological characteristics of the developed tumors were analyzed.

Histology and immunohistochemistry

The histological parameters were determined on formalin-fixed, paraffin-embedded, and hematoxylin-eosin (HE)-stained sections of the developed tumors. The averaged number of cell divisions was measured by counting the number of nuclei showing clear signs of mitosis in ten individual visual fields at high magnification. In addition, to assess the number of proliferating cells, formalin-fixed, paraffin-embedded sections were immunostained against the nuclear marker Ki-67 using a streptavidin-biotin-complex (SABC) three-step immunohistochemical technique.

RESULTS

1) The characteristic PKC isoform pattern of HaCaT keratinocytes changes during proliferation and differentiation

During the first phase of our experiments, using immunocytochemistry and Western blotting, we clarified that HaCaT cells express 7 isoforms of PKC: the cPKCα and β; the nPKCδ, ε, η, and θ; and the aPKCζ. We, however, were unable to detect any expression of aPKCλ/ι and cPKCγ. We have also shown that the levels of individual PKCs differentially altered during culturing. The expressions of certain isoforms (cPKCα and β; nPKCη and θ, aPKCζ) did not alter significantly during proliferation and high cell density-induced differentiation. In contrast, the expression of nPKCδ reached marked (and monotonously increasing) levels only in the differentiating cultures, whereas the expression of nPKCε increased parallel with proliferation but, then, the isoform practically disappeared from the differentiating (post-confluent) cultures. Using confocal microscopy analyses on cells immunostained for the different PKC isozymes, however, we could not detect significant changes in subcellular localization of any of the isoforms during proliferation and differentiation of the cells.

2) The expression of differentiation markers and the resting $[Ca^{2+}]_i$ change during proliferation and differentiation of HaCaT keratinocytes

We also investigated the expression of keratinocyte differentiation markers. The level of keratin (K) 10 reached its maximal value upon confluence and days 1-2 of post-confluence (a rather early marker), whereas the expressions of involucrin (INV), filaggrin (FIL), and transglutaminase-1 (TG) were most dominant in the late post-confluent cultures (late/terminal differentiation markers). The level of the hyperproliferation-associated marker K17

insignificantly changed during culturing. We have, furthermore, shown that the $[Ca^{2+}]_i$, which can also be regarded as a differentiation marker, monotonously decreased with increasing proliferation tendency of the pre-confluent cells, whereas it gradually increased in the differentiating cultures.

3) PMA inhibits cellular proliferation, increases $[Ca^{2+}]_i$, and alters the expressions of differentiation markers in HaCaT cells

In the next phase of our experiments, we investigated the effects of PMA on cellular functions of HaCaT cells. On pre-confluent (thus proliferating) cells we found that PMA, possessing characteristic ("U-shaped") dose-response features, inhibited the proliferation of HaCaT cells (the most effective doses were 10 and 100 nM PMA). This effect was specific via the PKC system since GF109203X, a PKC inhibitor, significantly prevented the inhibitory effect of PMA on cellular proliferation. On differentiating cultures, the application of the phorbol ester inhibited the expressions of early differentiation markers K10 and K17, whereas the levels of late-terminal markers INV, FIL, and TG were gradually and significantly increased by PMA. Finally, we have also shown that PMA increased the resting $[Ca^{2+}]_i$ in the pre-confluent cells which also argues for that PMA initiates terminal differentiation in HaCaT keratinocytes.

4) The PMA-induced cellular effects on proliferation and differentiation are differentially mediated by certain PKC isoforms

Experiments performed on proliferating HaCaT keratinocytes revealed that PMA differentially altered (down-regulated) the various PKC isoforms exerting distinct dose-response relationships. Actually, most of the sensitive isozymes (i.e., cPKCs and nPKCs) but not the "atypical" PKCζ possessed changes in their expressions, although the degrees of down-regulation were essentially different. There were isoforms (PKCα, β, ε, and θ), which completely disappeared from the cells upon PMA treatment, although different

phorbol ester concentrations were needed to cause down-regulation. In contrast, the down-regulation of PKCδ was significant (40-50%), although the isoform possessed marked expression levels in the PMA-treated cultures. Finally, we found a very little (max. 20%) down-regulation of PKCη upon PMA treatment.

On differentiating HaCaT keratinocytes, similarly to that found in proliferating cells, PMA differentially affected the expressions of the various PKC isoforms. However, it was of greatest importance that the PKC isoform population, which showed sensitivity to PMA in the differentiating HaCaT cells, was essentially different from that seen in the proliferating ones. PMA, in contrast to the proliferating cells, did not cause any measurable down-regulation of PKCδ and η. Furthermore, although PKCα and θ were also down-regulated by PMA in the differentiating cells, the dose-response curves of PMA to induce down-regulation of these isoforms were different on the two cell populations. Namely, PKCα was more readily down-regulated by PMA in the differentiating cultures, whereas the expression of PKCθ decreased more in the proliferating cells upon PMA treatment. Finally, cPKCβ showed very similar sensitivities to the action of PMA in both proliferating and differentiating cultures.

5) Effects of PKC inhibitors on proliferation and differentiation of HaCaT keratinocytes

By investigating various PKC inhibitors we have shown that GF109203X, an inhibitor of the cPKC and nPKC isoenzymes, significantly suppressed the expression of the late differentiation markers INV, FIL, and TG in a dose-dependent manner; in contrast, however, it did not modify the proliferation of the cells. We have also found that Gö6976, an inhibitor of cPKCα and β, markedly inhibited the proliferation of the cells in a dose-dependent manner whereas it induced a very similar pattern of changes in the expression of differentiation markers when compared to the effect of GF109203X. Finally, the

inhibition of nPKCδ by the specific inhibitor Rottlerin – very similarly to the action of GF109203X and Gö6976 – dose-dependently inhibited the expression of the differentiation markers (INV, FIL, TG) whereas stimulated cellular proliferation.

6) Effects of overexpression of PKC isoforms on morphology of HaCaT cells

In the next phase of our experiment, using recombinant techniques, we have constructed HaCaT keratinocytes stably overexpressing certain PKC isoforms. We found that the overexpression of the PKC isoforms differentially affected the morphology of HaCaT keratinocytes. The characteristic cobblestone morphology of control (empty vector-transfected) HaCaT cells was dramatically changed by the overexpression of nPKCε; these cells exhibited a fibroblast-like, spindle-shaped phenotype. In addition, cells overexpressing cPKCβ also possessed elongated cell bodies and formed more dense islets during culturing. The overexpression of nPKCδ and cPKCα did not result in dramatic morphological changes; possibly the nPKCδ overexpressing HaCaT cells displayed a more cobblestone appearance.

7) Effects of overexpression of PKC isoforms on the proliferation and differentiation of HaCaT cells

Experiments investigating the *in vitro* cellular functions of the recombinantly modified cells revealed that the overexpression of the PKC isoforms markedly altered growth of the cells. The overexpression of cPKCα and of nPKCδ decreased the proliferation and doubling time of HaCaT cells and suppressed the maximal saturation densities of the cultures. Conversely, keratinocytes overexpressing the cPKCβ and nPKCε exhibited higher proliferation rates, accelerated doubling times, and elevated saturation densities.

Using quantitative Western blot we have, furthermore, shown that in (hypoproliferative) cells overexpressing the cPKCα and nPKCδ isoenzymes the levels of the late-terminal differentiation markers (INV, FIL, TG) significantly increased, whereas in (hyperproliferative) keratinocytes overexpressing the cPKCβ and nPKCε isoforms the levels of the differentiation markers decreased compared to those of the control HaCaT cells.

8) Effects of overexpression of PKC isoforms on the apoptosis of HaCaT cells

As determined by annexin V-based flow cytometry analysis, there were no major differences in the level of basal apoptosis for control and cPKCβ and nPKCε overexpressing HaCaT cells. In contrast, the basal apoptotic rates in the nPKCδ transfectants and, yet to lesser extent, in the cPKCα overexpressers were higher than in the control cells. In addition, the overexpression of PKCα and δ increased the susceptibility of cells to apoptosis induced by 10 μM $1\alpha,25(OH)_2D_3$ or 50 nM TNFα, whereas the PKCβ and ε overexpressers showed less sensitivity to the action of these agents than did the control cells.

9) Effects of overexpression of PKC isoforms on tumorigenicity in SCID mice

Finally, we investigated the behavior of PKC overexpressing cells in assays for tumor formation and *in vivo* growth. SCID mice were injected with cell suspensions of HaCaT cells overexpressing different PKC isoforms (along with control keratinocytes) and, after 30 days, the tumors, which developed were characterized. Histological analysis of tumors revealed that control HaCaT cells formed basal cell-enriched tumors with expansive growth properties at the periphery and with intense maturation and differentiation (formation of keratin islets, dyskeratotic cells, sometimes cyst formation) at the center. The relative sizes of the proliferating and differentiating fields were approximately the same. The injection of HaCaT cells overexpressing the various PKC isoforms

generally did not change the major histological characteristics of the tumors. Namely, all tumors maintained the expansive (i.e., non-infiltrative, benign) growth characteristics and histological features of peripheral proliferation and central differentiation. However, of greatest importance, we found marked differences in the average size of the tumors, the number of dividing cells, and the relative ratio of the proliferating and differentiating parts on the histological sections. The overexpression of nPKCε and cPKCβ resulted in increased tumor growth, as reflected by the markedly increased tumor sizes and number of dividing cells. Moreover, the relative ratio of the proliferating to the differentiating parts also increased in these tumors. In contrast, tumors initiated by cPKCα and nPKCδ overexpressers possessed suppressed averaged tumor sizes, decreased numbers of mitosis, and an increased dominance of the differentiating part. These findings were further strengthened by immunohistochemical staining of Ki-67 positive (thus proliferating) cells on the tumor sections.

DISCUSSION

1) The PKC isoforms pattern of HaCaT keratinocytes and its alteration during proliferation and differentiation

During our experiments, we first described the PKC isoenzyme pattern of HaCaT keratinocytes. We have shown that HaCaT keratinocytes expressed several PKC isoforms: the cPKCα and β; the nPKCδ, ε, η, and θ; and the aPKCζ. It is generally accepted that NHEK express PKCα, δ, ε, η, and ζ, however, the existence of other PKC isoforms (namely PKCβ, γ, and μ) was also documented. Since the only isoform that has never been identified in NHEK but is expressed in HaCaT cells is the PKCθ, we can conclude that the characteristic PKC isozyme pattern of HaCaT keratinocytes is similar to that of NHEK.

However, when we investigated the changes in the level of expressions and subcellular localization of the various PKC isoforms with proliferation and differentiation, some marked differences between NHEK and HaCaT cells were observed. In HaCaT cells, we found differential changes in the expression patterns of the isoforms during culturing: the expression of nPKCδ increased with proliferation and differentiation whereas the expressions of nPKCε showed biphasic pattern: it increased in the proliferating and declined (disappeared) in the differentiating cultures. The expressions of the other isoforms were essentially the same during culturing. In NHEK *in vitro*, the expression levels of the existing PKCs did not change significantly during high cell density-induced differentiation, although *in vivo* the nPKCη was abundantly expressed only in the well-differentiated layers. Similarly, in NHEK and mouse keratinocytes induced to differentiate by elevating extracellular calcium concentration marked changes in expression and activity of the PKC were observed during differentiation.

A more prominent difference between HaCaT cells and NHEK was found when we examined the subcellular localization of the PKC isoforms during culturing. As was revealed by confocal microscopy, there were no measurable alterations in subcellular localization with any of the existing isoforms in HaCaT cells, which finding was in a marked contrast to those describing that, both in NHEK and mouse keratinocytes, certain PKC isozymes translocated upon differentiation. This difference may be due to the nature of the existing PKC isoforms in HaCaT cells or to the different experimental design to follow intracellular translocation.

2) The activation of the PKC system by PMA induces terminal differentiation

When investigating the effect of a general PKC activator PMA, we found that the phorbol ester, by specifically acting on sensitive PKC isoforms (see below), inhibited cellular growth and – identically to those described on NHEK – induced terminal differentiation. We have also shown that PMA exerted its cellular effects on proliferating HaCaT cells (to inhibit proliferation) and on differentiating cells (to induce terminal differentiation) by differentially affecting the levels (i.e., down-regulating) of various PKC isoforms. In the proliferating cells, PMA down-regulated practically all of the sensitive cPKC and nPKC isoforms although the degrees of down-regulation and sensitivities of the various isoforms to PMA were usually different. In the differentiating cells, however, PKCα, β, and θ were down-regulated by the phorbol ester (again, showing mostly different degrees and dose-response characteristics of down-regulation) whereas PKCδ and η were insignificantly modified by PMA. In addition, among those isoforms that were down-regulated both in the proliferating and differentiating cells by PMA, PKCα was more readily down-regulated in the differentiating cells whereas PKCθ showed more prominent down-regulation in the proliferating cells. It appears, therefore, that the

sensitivities of most of the PKC isoforms to the action of PMA altered with the onset of high cell density-mediated differentiation reflecting possible changes in their activities in regulating the process.

If we assume that the various PKC isozymes of a given cell may differentially and sometimes antagonistically act in regulating certain cellular processes, the differential sensitivities of the PKCs to PMA may also explain the different dose-response relationships of PMA to inhibit proliferation and to modify the expressions of the differentiation markers. For example, in the proliferation experiments, the “U-shaped” dose-response curve of PMA (i.e., 100 nM PMA was more effective than 1 μM PMA in inhibiting proliferation) can be explained by that the two different doses resulted in markedly different PKC isozyme patterns by differentially down-regulating the isoforms. Therefore, if the activity of a certain isoform that would inhibit proliferation was decreased and/or the activity of another that would promote cellular growth was increased by 1 μM PMA (and not by 100 nM PMA), the result would be a less pronounced growth inhibitory effect of the phorbol ester at the higher concentration.

3) Specific roles of PKC isoenzymes in the regulation of proliferation, differentiation, apoptosis, and tumorigenesis of HaCaT cells

Our molecular biological experiments provided further evidence for the isoform-specific roles of the PKC isoenzymes. The cPKCα isoform, one of the most studied PKC isoforms in keratinocyte biology, was previously shown to play a central role in the calcium- and high cell density-induced terminal differentiation program in mouse and human keratinocytes. In good accord with these reports, we have shown that the overexpression of cPKCα promoted differentiation and the sensitivity for apoptosis inducing agents but inhibited proliferation and tumor growth. These findings strongly argue for that, similarly

to its behavior in NHEKs, cPKCα may play a central role in the positive regulation of differentiation and negative regulation of growth of HaCaT keratinocytes.

Comparison of data obtained with cells overexpressing cPKCα (i.e., stimulation of apoptosis and differentiation, inhibition of growth) with those obtained using Gö6976, the inhibitor of the cPKC isoforms (i.e., inhibition of both proliferation and differentiation) suggested that cPKCβ (another cPKC isoform in HaCaT cells that presumably was also inhibited by Gö6976) may oppositely regulate the above processes. Indeed, the proliferation and tumorigenic activity of cells overexpressing cPKCβ was markedly increased whereas their differentiation and apoptotic tendencies were suppressed when compared to the control HaCaT cells. These findings were in good accord with previous data stating that the expression of cPKCβ is altered in psoriasis (one of the most frequent hyperproliferative skin diseases) where the sophisticated balance of keratinocyte proliferation and differentiation is impaired.

During the investigation of the novel PKC isoenzymes, a strikingly similar phenomenon was observed. The nPKCε has been extensively documented as a key positive regulator of cellular proliferation in various cell types. It was also shown that the overexpression of nPKCε leads to pathological proliferation (hyperproliferative transformation) and that down-regulation or overexpression of the dominant negative mutant of the enzyme result in inhibition of proliferation and induction of differentiation. In addition, very recently, the promoting role of nPKCε in skin tumor formation in transgenic mice was also reported. Finally, in the current study we have shown that, as in NHEK, nPKCε is almost exclusively expressed in the proliferating but not in the differentiating HaCaT cells and, furthermore, that the growth rate and tumorigenicity of cells overexpressing nPKCε dramatically increased whereas the differentiation and apoptotic capacities decreased. These data

unambiguously argue for the positive regulatory role of this isoform in *in vitro* and *in vivo* growth of HaCaT keratinocytes.

Opposite findings were obtained regarding nPKCδ; i.e., this isoform functioned as a positive regulator of differentiation and apoptosis with a parallel inhibitory action on cellular and tumor growth. Several groups have reported that the activation or overexpression of nPKCδ in keratinocytes inhibited proliferation, initiated the differentiation program, and mediated the apoptotic effect of several inducers in NHEK. In addition, it was shown that malignant transformation (i.e., increased proliferation tendency) of HaCaT keratinocytes by Ha-ras overexpression resulted in the disappearance of nPKCδ from the cells and that the treatment of control HaCaT cells by Rottlerin induced an altered (proliferating) phenotype. Consistent with these literature data, our findings further argue for the positive and central role of nPKCδ in the initiation and development of differentiation and apoptosis.

Finally, we should note that, to the best of our knowledge, this is the first demonstration of the tumor inducing properties of HaCaT cells in SCID mice. In contrast to data obtained in nude mice, where control HaCaT cells formed characteristic cystic granules (which regressed after few weeks), HaCaT keratinocytes induced expansively growing benign tumors in SCID animals which were histologically very similar to those developed by injecting benign ras-transfected HaCaT cells into nude mice. These data argue that, similar to the growth properties of other tumorigenic cell types, the tumor inducing capabilities of HaCaT cells are more profound in SCID mice than in nude mice. Nevertheless, since the overexpression of none of the PKC isoforms (not even of the hyperproliferative cPKCβ or nPKCε) resulted in malignant transformation (in contrast to malignant ras transfection), it appears that although certain PKC isoforms stimulate *in vitro* and *in vivo* growth of HaCaT keratinocytes, their

constitutive presence alone is not enough for malignant transformation of the cells.

In summary we can conclude that the isoform-specific roles of certain cPKC and nPKC isoenzymes enrolled in this study in the regulation of *in vitro* and *in vivo* growth, differentiation, and apoptosis of human HaCaT keratinocytes are very similar to that previously described on NHEK supporting the relevance of our findings for complete normal human epidermal cells.

SUMMARY

We investigated the roles of protein kinase C (PKC) isoenzymes in regulation of cellular functions of human HaCaT keratinocytes. We found that the cells possessed a characteristic PKC isoform pattern (cPKCα, β; nPKCδ, ε, η, and θ; aPKCζ) which was significantly altered during proliferation and differentiation of the cells. It was also proven that the general PKC activator phorbol 12-myristate 13-acetate, by differentially acting on certain isoforms, inhibited HaCaT keratinocyte proliferation but induced terminal differentiation. Using various PKC inhibitors we have also shown that the joint inhibition of cPKC and nPKC isoforms (using GF109203X) did not modify proliferation although it inhibited terminal differentiation. As a marked contrast, the sole inhibition of cPKCs (using Gö6976) significantly suppressed both cellular proliferation and differentiation of the cells, whereas the inhibition of nPKCδ (using Rottlerin) increased proliferation but inhibited differentiation. In our molecular biological experiments, in which we established HaCaT keratinocytes stably overexpressing various PKC isoforms, we found that the overexpression of cPKCα and nPKCδ dramatically inhibited *in vitro* cellular proliferation and *in vivo* tumor growth in immunodeficient (SCID) mice, whereas these interventions stimulated differentiation and apoptosis of the keratinocytes. Conversely, the overexpression of cPKCβ and nPKCε stimulated *in vitro* and *in vivo* growth but inhibited cellular differentiation and apoptosis. These data strongly argue for the specific yet antagonistic roles of certain cPKC and nPKC isoenzymes in regulation of cellular processes of human HaCaT keratinocytes.

PUBLICATIONS

THE THESIS WAS BUILT ON THE FOLLOWING PUBLICATIONS:

1) *Papp H., Czifra G., Lázár J., Gönczi M., Csernoch L., Kovács L., and Bíró T.* (2003): Protein kinase C isozymes regulate proliferation and high cell density-mediated differentiation in HaCaT keratinocytes. *Exp Dermatol.* 12:811-824. IF: 2,303
2) *Papp H., Czifra G., Bodó E., Lázár J., Kovács I., Aleksza M., Juhász I., Ács P., Sipka S., Kovács L., Blumberg P.M., and Bíró T.* (2003): Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. *Cell. Mol. Life Sci.* 61:1095-1105. IF: 5,259

OTHER PUBLICATIONS:

3) *Boczán J., Bíró T., Czifra G., Lázár J., Papp H., Bárdos H., Ádány R., Mechler F., and Kovács L.* (2001): Phorbol ester treatment inhibits proliferation and differentiation of cultured human skeletal muscle satellite cells by differentially acting on protein kinase C isoforms. *Acta Neuropathol.* 102:55-62. IF: 2,283
4) *Gönczi M., Papp H., Bíró T., Kovács L., and Csernoch L.* (2002): Effect of protein kinase C on transmembrane calcium fluxes in HaCaT keratinocytes. *Exp. Dermatol.* 11:25-33. IF: 2,303

CITABLE ABSTRACTS:

1) *Bíró T., Papp H., Lázár J., Czifra G., and Kovács L.* (2000): Distinct roles of protein kinase C isozymes in regulating proliferation and differentiation of HaCaT keratinocytes. *J. Invest. Dermatol.* 115(3):548
2) *Bíró T., Papp H., Czifra G., Lázár J., and Kovács L.* (2001): Endogenous activation of protein kinase C regulates proliferation and differentiation in HaCaT keratinocytes. *J. Invest. Dermatol.* 117(2):502
3) *Bíró T., Papp H., Bodó E., Lázár J., Czifra G., and Kovács L.* (2002): Modification of protein kinase C alters proliferation and differentiation of immortalized human keratinocytes. *Rev. Oncol.* 4(1):82
4) *Szentandrássy N., Gönczi M., Papp H., Lázár J., Kovács L., Bíró T., and L. Csernoch* (2002): Stretch activated channels and their regulation by protein kinase C in HaCaT keratinocytes. *J. Invest. Dermatol.* 119(3):746

5) *Lázár J., Czifra G., Papp H., Marincsák R., Papp L., Kovács L., and Bíró T.* (2002): Protein kinase C regulates the sensitivity of recombinant rat vanilloid receptor-1 to capsaicin. *Acta Physiol. Hung.* 89(1-3):13
6) *Papp H., Bodó E., Lázár J., Czifra G., Kovács L., and Bíró T.* (2002): Effects of pharmacological modification of protein kinase C on proliferation and differentiation of human HaCaT keratinocytes. *Acta Physiol. Hung.* 89(1-3):348
7) *Czifra G., Papp H., Lázár J., Kern A., Kovács L., and Bíró T.* (2002): PKCδ mediates the growth promoting effect of IGF-I on cultured human skeletal muscle cells. *Acta Physiol. Hung.* 89(1-3):278
8) *Bíró T., Papp H., Bodó E., Lázár J., Czifra G., Kovács I., Gáspár K., Juhász I., and Kovács L.* (2003): Opposite roles of protein kinase C isoforms in regulating human HaCaT keratinocyte proliferation, differentiation, and tumor genesis. *J. Invest. Dermatol.* 121(1):218
9) *Czifra G., Papp H., Lázár J., Kern A., Kovács L., and Bíró T.* (2003): PKCδ mediates the growth promoting effect of IGF-I on cultured human skeletal muscle cells. *Neuromuscular Disord.* 13(7-8): 621
10) *Bíró T., Czifra G., Bodó E., Lázár J., Tóth I.B., Papp H., Kovács I., Juhász I., and Kovács L.* (2004): Cell and isoform specific roles of protein kinase C isoenzymes in regulating in vitro and in vivo proliferation of keratinocytes and skeletal muscle cells. *J. Invest. Dermatol.* 122(3):A21