THESES FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

DIFFERENTIAL ROLES OF PROTEIN KINASE C ISOENZYMES IN THE REGULATION OF IN VITRO AND IN VIVO GROWTH OF SKELETAL MUSCLE CELLS AND CHONDROCYTES

Gabriella Czifra

UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
MEDICAL FACULTY
DEPARTMENT OF PHYSIOLOGY

Debrecen
2006

INTRODUCTION

Skeletal muscle regeneration

The skeletal muscle possesses a remarkable *in vivo* and *in vitro* regeneration capacity which plays a key role in posttraumatic and postnecrotic muscle reconstitution seen e.g. in dystrophies or myopathies. The *in vivo* muscle regeneration is initiated from satellite cells which, upon specific stimuli, start proliferating (development of myoblast); their DNA incorporates to the cell nuclei of quiescent muscle cells, and then fuse and differentiate to form multinucleated muscle fibers. The mechanism of muscular regeneration can be well modeled *in vitro* by culturing skeletal muscle cells. Satellite cells isolated enzymatically and mechanically from muscle biopsy specimens proliferate, differentiate, and fuse to form multinuclear myotubes. Another possible *in vitro* method is to establish secondary cultures of skeletal muscle cell-derived cell lines in which similar differentiation processes can be initiated.

The mechanism of muscle regeneration can be exemplified by the following chain of events: activation of satellite cells may be induced by polymorphonuclear leukocytes (migrating to the place of muscle injury) and the cell-cell interactions (integrins, adhesion molecules) of satellite cells. In parallel, the hepatic growth factor produced by the muscle fibers and satellite cells stimulates proliferation and migration of cells. Afterwards, further proliferation, differentiation, and fusion of activated satellite cells is promoted and regulated by the autocrine and paracrine connections of numerous other growth factors and cytokines (e.g. interleukins).

Proliferation and differentiation of muscle cells is furthermore affected by various muscle-specific factors acting on distinct signaling mechanisms. The activity of these myogenic regulatory factors (which may lead to the production of muscle-specific proteins) is markedly modified by their phosphorylation

states which can be modulated by the signal transduction pathways coupled to the above growth factors and cytokines.

IGF-I act as a central regulatory molecule of skeletal muscle regeneration

Insulin-like growth factor-I (IGF-I), which bears a marked structure homology with insulin, is produced in numerous tissues including skeletal muscle and the satellite cells. It was unambiguously shown that IGF-I is an essential growth factor in muscle biology since it is required for the growth and development of the muscle. IGF-I stimulates gene expression, DNA and protein synthesis, different transport mechanisms, and migration, proliferation, and differentiation of cultured myogenic cells. It was also documented that the expression of IGF-I is increased in satellite cells, myoblast, myotube and muscle fibers of injured and atrophic skeletal muscles suggesting that the growth factor may stimulate regenerative processes in the muscle. This hypothesis was also strengthened by that the administration of recombinant human IGF-I or specific gene transfer therapies improved metabolism and function of impaired skeletal muscles of patients with muscle diseases.

It is generally accepted that IGF-I exerts its mitogenic cellular effects by acting on its one-transmembrane domain receptor, which bears tyrosine kinase activity. However, the participation of various signaling pathways and kinase systems is very controversially described on skeletal muscle cell types of different species. Namely, in mouse and rat skeletal muscle preparations (similarly to other cell types) the involvement of both the mitogen-activated protein kinase (MAPK) pathway and MAPK-independent signaling mechanisms, including the Akt/phosphatidylinositide 3-kinase (PI3-K) and the protein kinase C (PKC), were equally documented.

We possess, however, only very limited data about the IGF-I mediated responses of human skeletal muscle cells. Although (in the very few reports) it was documented that IGF-I stimulates the hypertrophy and fusion (hence

proliferation and differentiation) of myogenic cells in human cultures as well, we lack the exact description of the cellular mechanism underlying potential therapeutic applications

The protein kinase C isoenzymes

The PKC isoenzyme family is one of the key Ser/Thr-specific kinase systems. Up to date, at least 11 PKC isoenzymes were identified which, based on their activation mechanisms and structure characteristics, can be classified to four groups; the calcium- and phorbol ester-dependent "conventional" (PKCα, βI, βII, and γ; cPKCs); the calcium-independent "novel" (PKCδ, ε, η, and θ; nPKCs); the calcium- and phorbol ester-independent "atypical" (PKCζ, and λ/ι; aPKCs); and the unique PKCμ (or as recently named, PKD) groups.

PKC enzymes modulate a wide array of physiological regulatory processes. They play pivotal and central roles in the regulation of for example cellular proliferation and differentiation, the sequential events of apoptosis, or the cell-specific synthesis of various mediators (vasoactive substances, growth factors, cytokines).

Recent reports, however, also indicate that, besides the existing heterogeneity in their structural characteristics, activation, and cell-specific distribution, the regulation and biological roles of the PKC isoenzymes might also significantly differ from one another. It was also proven that not only may some PKC isoforms be active whereas others not for a given response (of great importance, for proliferation and differentiation), but different PKC isoenzymes may have antagonistic effects on the same cellular event.

The PKC isoforms and the skeletal muscle

The regulatory role of the PKC systems was documented in the skeletal muscle as well. It was shown that the modification of PKC activity elevated insulin-dependent glucose transport, stimulated vitamin D-induced calcium

uptake, and mimicked the effects of prostanoid to promote fusion of myoblasts. With respect to the specific roles of the isoenzymes, nPKCθ were implicated in mediating the complex effect of insulin to control muscle homeostasis whereas cPKCα and nPKCδ were shown to participate in the effect of tumor necrosis factor-α to inhibit insulin signaling. In addition, nPKCδ and aPKCζ were found to positively regulate glucose transport whilst aPKCζ and λ were documented to play a role in the regulation of exercise-related changes in metabolic and gene-regulatory responses of human skeletal muscle.

The PKC system also plays a role in the regulation of skeletal muscle cellular growth. In primary cultures of rat satellite cells, treatment with highly specific PKC inhibitors or with phorbol 12-myristate 13-acetate (PMA) did not significantly alter myogenic differentiation. In contrast, in cultured chick muscle cells, PMA modified the expressions of certain differentiation markers, selectively and reversibly inhibited the ongoing differentiation program, blocked DNA synthesis, and down-regulated cPKCα. In part similar to these findings, in normal human skeletal muscle cells, PMA inhibited proliferation and fusion of the cells in a dose-dependent fashion, suppressed the expression of the muscle-specific differentiation marker desmin, and (in already fused myotubes) inhibited myogenesis.

We, however, possess very limited, often debating, and (similarly to the above) species-dependent data about the specific roles of the PKC isoforms in proliferation and differentiation of skeletal muscle cells. According to our best knowledge, up to date only the cPKCα was introduced as a central promoter of cellular growth of cultured avian myoblasts whilst nPKCθ was suggested to promote differentiation of mouse and human skeletal muscles (although the overexpression of the isoforms in mouse muscle cells did not affect the fusion activity).

The hyaline cartilage – the key steps of chondrogenic differentiation

The hyaline cartilage is localized to various places (bone surface of the joints, visceral cartilage) in the vertebrate organisms. Cells of the "constant" cartilage are regenerated from chondrogenic stem cells located in the inner layer of the perichondrium whereas such processes are not found in the differentiated cartilages of the joint due to the lack of the perichondrium. During the embryonic life, the cartilage is developed from the mesechyme tissue. The proliferation and chondrogenic differentiation of stem cells, the acquisition of chondrocyte phenotype, and the establishment of the cartilage tissue are all strongly dependent on genomic factors ("master genes", see below), local micro-environmental agents (autocrine and paracrine growth factors, cytokines, extracellular matrix and adhesion molecules), and the pattern of surface membrane molecules (integrins, cytokine receptors) of the differentiating cells. Thus, the differentiation of the chondrocytes may be regulated by numerous factors (hormones, locally produced cytokines) of various origins. These processes can be well modeled using chondrogenic high-density (HD) chicken limb bud mesenchymal cultures.

The initial step of both *in vivo* and *in vitro* chondrogenic differentiation is the condensation of the cells to form prechondrogenic cell aggregates. These early cell-cell interactions are established by expression of adhesion molecules at the surface of chondrogenic cells and the appearance of numerous gap junctions. Besides, during the differentiation to mature chondrocytes, the cells start to express cartilage-specific extracellular matrix (ECM) molecules which unique microenvironment plays a key role in the development and maintenance of the final chondrocyte-specific phenotype.

Regulation of chondrogenesis – a putative role of the PKC system

One of the key "master" genes of the process of cartilage differentiation is the transcription factor Sox9. Moreover, it is also of great importance that the

differentiating cells produce a large amount of prostaglandin-E_2 which, by increasing intracellular cAMP, results in the activation of cAMP-dependent protein kinase A (PKA). It was shown that, during cartilage differentiation, the PKA can be activated by both cAMP and the bone morphogenic protein-2 (BMP). In addition, it was also proven that, in the chondrogenic cells, PKA phosphorylates the Sox9 which, in turn, activates genes of two central ECM molecules, i.e. the type II collagen and core protein of aggrecan. Another putative target of PKA is the "cAMP responsive element binding protein" (CREB) family of transcription factors. It was shown that phosphorylation (hence activation) of members of the CREB-family is increased not only by cAMP but also by one of the most important "chondrogenic" cytokine family, the transforming growth factor-β group (e.g. BMPs) which promotes mesenchymal condensation.

It was, however, also revealed that, in chondrocytes, CREB can be additionally phosphorylated by signaling pathways of the PKC system which proposed the potential role of the PKC-coupled mechanisms in the regulation of cartilage differentiation. Initially, PKC was suggested to inhibit differentiation since staurosporin (as a PKC inhibitor) stimulated the chondrogenesis; later, however, it was shown that this was due to other effects of the inhibitor. It was also documented that the general inhibition of PKC activity indeed resulted in suppression of cartilage differentiation. Intriguing findings furthermore suggested that the phosphorylation level of the MAPK Erk-1 was elevated in parallel with the inhibition of the PKC system which suggested that inhibition of PKC might lead to the inhibition of differentiation via the activation of Erk-1. However, these sporadic data – although unambiguously argued for the importance of the PKC system in chondrogenic differentiation – did not provide insight to the details of PKC-dependent processes. In addition, we practically lack the description of specific roles of certain PKC isoenzymes in the processes of chondrogenesis.

AIMS OF THE STUDY

In our experiments, the following experimental goals have been defined:

1. First, we intended to characterize the effects of IGF-I on *in vitro* growth, fusion, and differentiation of primary human skeletal muscle cells.
2. We then investigated the (potential) contribution of various signaling pathways in the development of cellular effects of IGF-I.
3. On the C2C12 myoblast cell line, by expanding our experimental techniques with pharmacological (selective and specific PKC inhibitors) and molecular biology approaches, we also measured the signaling pathways. As a part of these efforts, we established C2C12 myoblasts overexpressing certain PKC isoforms and then investigated the processes of *in vitro* proliferation, fusion, and differentiation, and the morphological characteristics
4. In our *in vivo* experiments, by injecting the PKC overexpresser C2C12 cells to immunodeficient mice and hence inducing tumors, we analyzed the roles of PKC isoenzymes in the regulation of tumorigenic mechanisms.
5. In the last phase of our study, using chondrogenic high-density (HD) chicken limb bud mesenchymal cultures, we investigated the roles of PKC isoforms in the regulation of *in vitro* chondrogenesis. We first characterized the PKC isoform pattern of the cultures and followed the expression changes during the process of chondrogenesis.
6. In addition, we intended to measure the possible modifications in PKC enzyme activities as the function of differentiation state, with special emphasis on the PKCμ isoform. Eventually, we investigated the cartilage formation in the presence of the PKCμ inhibitor resveratrol.

MATERIALS AND METHODS

Cell culturing

During the culturing of human skeletal muscle cells, muscle samples obtained from orthopedic surgery were subjected to combined enzymatic digestion and the cells suspension was cleared from fibroblasts using magnetic cell sorting. The satellite cell-enriched fraction was then cultured in Ham F-12 solution supplemented with 5% fetal calf serum (FCS) and 5% horse serum (HS) at 37 ^{o}C and 5% CO_2. At day 3, the medium was changed to Dulbecco's Modified Eagle's Medium (DMEM) which, due to its low 2% FCS and 2% HS content, promoted fusion and differentiation of myoblasts.

The C2C12 mouse rhabdomyoma cell line was cultivated at 37 °C-on and 5% CO_2 in DMEM supplemented with 15% FCS and antibiotics.

The HD mesenchymal chondrogenic cells were isolated from distal parts of the limb buds of Ross hybrid chicken embryos of Hamburger–Hamilton stages 22-24 and then the cells were cultured in Ham F-12 medium supplemented with 10% FCS and antibiotics.

Investigation of cellular proliferation

Growth of human satellite cell cultures was monitored by evaluating the morphological signs and by counting the number of nuclei (growth curves, 5 visual field per well).

In the case of control and PKC overexpresser C2C12 cell line, proliferation was measured by a colorimetric bromo-deoxyuridine (BrdU) assay kit. The determination is based on that the thymidine analog BrdU incorporates to the DNA of the proliferating cells which can be colorimetrically measured by the peroxidase enzyme coupled to the Fab-fragment of an anti-BrdU antibody and tetramethybenzidine substrate.

In the case of the PKC overexpressers, doubling times and maximal cell densities were also determined. The following equation was used to calculate the 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.

Proliferation activity of the HD chondrogenic cultures were determined by measuring radioactive ^{3}H-thymidine incorporation.

PKC vectors

PKC constructs were engineered as described previously. Briefly, the cDNA sequences of PKCα, β, δ, and ε and of the kinase (dominant)-negative mutant of nPKCδ (DN-nPKCδ) were subcloned into a metallothionein promoter-driven eukaryotic expression vector (MTH). The vector sequence encodes a C-terminal PKCε-derived 12 amino acid tag (ε-tag) and attaches it to the end of the PKC proteins.

Establishment of recombinant PKC overexpresser C2C12 cells

C2C12 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, or DN-nPKCδ using Superfect anionic detergent. Transfected cells were then selected in DMEM containing 825 μg/ml geneticin for 12-18 days, single colonies were isolated, and these cultures were cultivated in supplemented DMEM containing 500 μg/ml G418. The efficacy of recombinant overexpression was monitored by Western blotting and kinase assay.

Western blot analysis

Cells were homogenized in ice-cold lysis buffer, disrupted by sonication, and protein content of samples was measured. Then, after SDS-PAGE, the

separated proteins were transferred to nitrocellulose membranes and were immunostained using appropriate primary and secondary antibodies. The immunoreactive bands were visualized by a chemiluminescent kit whereas detection was performed with either a light-sensitive film (Fujifilm) or a LAS 3000 darkbox. Quantitative determination of expression was assessed by a densitometer and an appropriate software.

PKC activity (kinase) assay

To measure the PKC activity, transfected C2C12 cells were homogenized in lysis buffer and the kinase activity of the cell lysates was determined using Histone III or the 20 kDa light chain of smooth muscle myosin (MLC20), as substrates. Activity was determined by measuring the incorporation of radioactive γ-^{32}P-ATP.

The PKC activity of chondrocytes was also defined by the above protocol. The assessment of PKCμ activity was, however, different since we determined the incorporation of radioactive γ-^{32}P-ATP to the PKCμ-specific substrate syntide-2.

Immunocytochemistry and confocal microscopy

The changes in subcellular localization of PKC isoforms (as an accepted sign of activation) upon IGF-I treatment was detected by confocal microscopy following immunolabeling. Cells were fixed in acetone and, after treating with blocking solution, were incubated with the appropriate anti-PKC antibodies. Then staining was continued using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Cells were finally covered by DAPI and images were obtained using a confocal microscope.

Metachromatic staining and light microscopy analysis of chondrogenic cultures

The HD cultures were fixed by the 4:1 mixture of 40% formaldehyde and absolute alcohol and then were stained with a 0.1% aqueous DMMB dissolved in 3% acetic acid. After DMMB staining at low pH, the cartilage matrix, due to its high proteoglycan content, possesses a string red metachromatic color, whereas the intensity of the orthochromatic blue color is negligible (under these circumstances).

Assessment of tumorigenesis in SCID mice

Cells overexpressing the various PKC isoforms at a density of 1-2 x 10^6 viable cells/200 μl were injected intradermally to Severe Combined Immuno Deficiency (SCID) mice and observed over a period of 30 days. Animals were finally euthanized and the averaged three-dimensional size and histological characteristics of the developed tumors (four-five animals for each group) were analyzed.

Histology and immunohistochemistry

The histological parameters as well as the number of cell divisions of the developed tumors were determined on formalin-fixed, paraffin-embedded, and hematoxylin-eosin (HE)-stained sections. To assess the number of proliferating cells, sections were immunostained against the nuclear marker Ki67 using a streptavidine-biotin-complex (SABC) three-step immunohistochemical technique.

RESULTS

On primary human skeletal muscle cells, the effect of IGF-I is mediated nPKCδ, whereas the MAPK and PI3-K pathways do not participate in the development of the cellular action

In the first phase of our experiments, we investigated the participation of signaling pathways in the effect of IGF-I on human skeletal muscle cells. IGF-I stimulated the growth of the cells in dose- and time-dependent manners. We have additionally shown that IGF-I also increased the fusion activity (started at day 5) and the expression of the muscle-specific differentiation marker desmin. These data suggested that IGF-I promoted both the early (proliferation) and late (fusion and differentiation) events of *in vitro* regeneration in human skeletal muscle cultures.

It was also revealed that the inhibition of the MAPK or PI3-K pathways did not modify the effect of IGF-I to stimulate proliferation and differentiation. In addition, IGF-I did not affect the phosphorylation state of the MAPK Erk-1/2 (as a sign of potential activation). In contrast, GF109203X (the general inhibitor of the PKC system) significantly suppressed cellular proliferation and the expression of the differentiation marker. These findings on primary human skeletal muscle cells argued for that, unlike the MAPK Erk-1/2 and PI3-K signaling mechanisms, certain member(s) of the PKC system participate in the development of the cellular effects of IGF-I.

Investigation of this argument revealed that the inhibition of cPKC isoforms expressed in human skeletal muscle cells (i.e., cPKCα and γ) by the selective inhibitor Gö6976 did not affect the action of IGF-I. We have, however, found that Rottlerin, the specific inhibitor of nPKCδ (at such low doses which alone did not modify the control growth and differentiation of the cells), fully suspended the cellular effect of IGF-I. Finally, we have shown that IGF-I

induced the selective translocation of nPKCδ from the cytoplasm to the nucleus and nuclear membrane (reflecting its potential activation) whereas it did not modify the subcellular localization of the other isoforms. These results strongly argued for the central and exclusive involvement of nPKCδ in mediating the cellular effect of IGF-I on human skeletal muscle cells.

On C2C12 myoblast, besides nPKCδ, the MAPK pathway is also involved in mediating the cellular effects of IGF-I

In the next phase, the above experiments were also repeated on mouse C2C12 myoblast which represent a proper model of human cultured skeletal muscle cells. IGF-I stimulated growth and fusion activity of C2C12 cells as well as the expression of the muscle-specific differentiation marker desmin. We have additionally shown that – also similarly to findings on human muscle cells – the effect of IGF-I was fully abrogated by GF109203X (a general inhibitor of the PKC pathway) whereas inhibition of the PI3-K pathway had no effect. However, in contrast to our data obtained on human muscle cells, the MAPK Erk-1/2 inhibitor PD098059 significantly yet not completely (by 30-40 %) prevented the action of IGF-I. Furthermore, we have shown that IGF-I treatment stimulated the phosphorylation of MAPK Erk-1/2. These data suggested that on C2C12 cells, besides the PKC system, the MAPK signaling pathway may also participate in the development of the effect of IGF-I.

To further assess the exact involvement of these signaling mechanisms, we subsequently measured the roles of certain PKC isoforms. We found that C2C12 myoblast possess 6 PKC isozymes: the cPKCα and β, the nPKCδ, η, and θ, and the aPKCζ. We have also shown that – similarly to human muscle cells – the selective nPKCδ inhibitor Rottlerin fully prevented the mitogenic effect of IGF-I whereas the inhibition of the cPKC isoforms (i.e., cPKCα and β in these cells) possesses negligible effect. Moreover, IGF-I remarkably and selectively

elevated the degree of tyrosine phosphorylation of nPKCδ which may reflect its activation of the isoform.

These data strongly supported our idea that nPKCδ may play a key role in the IGF-I-induced cellular processes on C2C12 myoblasts as well. To further analyze this hypothesis, we have established such C2C12 cultures which stably overexpress the constitutively active form of nPKCδ or its kinase (dominant) negative mutant form (DN-PKCδ). Importantly, C2C12 cells overexpressing PKCδ possessed a markedly accelerated proliferation rate when compared to the growth of control C2C12 cells. Actually, overexpression of PKCδ resulted in a very similar growth acceleration to that seen when control C2C12 cells were treated by IGF-I. In addition, IGF-I treatment of PKCδ overexpressers resulted in no further increase of proliferation, suggesting that the constitutive activation of nPKCδ „mimicked" the mitogenic effect of IGF-I on these cells.

We have also shown that the overexpression of DN-PKCδ dramatically suppressed the proliferation of the cells when compared to the growth of the control myoblasts. In addition, IGF-I was unable to induce any growth stimulation on these cells further arguing for that PKCδ is an obligatory element of the signaling pathway evoked by IGF-I.

PKCδ is an upstream regulator of the MAPK pathway in mediating the action of IGF-I on C2C12 myoblasts

The above results obtained with the overexpressers (i.e., PKCδ is a crucial mediator of the IGF-I signaling), along with those findings that inhibitor of PKCδ completely prevented whereas inhibition of the MAPK pathway only partially suspended the effect of IGF-I, argued for a possible relationship between PKCδ and the MAPK signaling. To investigate this hypothesis, we have shown that the presence of the specific PKCδ inhibitor significantly suppressed the IGF-I-induced phosphorylation of Erk-1/2 suggesting that the

effect of IGF-I to stimulate the phosphorylation of Erk-1/2 is PKCδ-dependent. Furthermore, the accelerated proliferation of nPKCδ overexpressers was partially yet significantly (approximately by 35-40 %) inhibited by the MAPK inhibitor PD098059. These data strongly argue for the intimate relationship between PKCδ and the MAPK-coupled signaling and introduce PKCδ as an „upstream" activator of the MAPK pathway in mediating the cellular effects of IGF-I on C2C12 myoblasts (i.e., its preceding activation is compulsory for the activation of the MAPK pathway).

Certain PKC isoforms differentially regulate the proliferation and the expression of the differentiation marker desmin in C2C12 myoblasts

In the next phase of our experiments, we have performed the recombinant stale overexpression of other PKCs (cPKCα and β, and nPKCε), and then measured the possible alteration in the growth properties of these cells. The overexpression of the various PKC isoenzymes differentially affected the growth of the cells. The overexpression of "conventional" cPKCα and β markedly decreased the proliferation of C2C12 cells whereas transfection with nPKCε resulted in insignificant changes in the growth rate. Conversely, confirming the aforementioned findings, myoblasts overexpressing the nPKCδ exhibited dramatically higher proliferation rates compared to the control (empty vector transfected) cells. Furthermore, in cells overexpressing the cPKCα and β isoenzymes, the levels of the differentiation marker increased, whereas in myoblasts overexpressing the nPKCδ isoforms the levels of the desmin remarkably decreased compared to those of the control C2C12 cells (data obtained with nPKCε overexpressers, again, revealed no differences).

Moreover, Gö6976, an inhibitor of the cPKC isoforms (i.e., the cPKCα and β in C2C12 cells) stimulated the proliferation of the cells in a dose-dependent manner. In contrast, the nPKCδ inhibitor Rottlerin dose-dependently

inhibited cellular growth. Furthermore, the overexpression of DN-nPKCδ resulted in a dramatically suppressed cellular proliferation rate and prolonged doubling time. These findings strongly supported that cPKCα and β are negative whilst nPKCδ is a positive regulator of proliferation in C2C12 myoblasts.

Overxepression of PKC isoenzymes induce different tumors 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 various C2C12 myoblasts and, after 30 days, the tumors, which developed were characterized. As revealed on histological sections, control C2C12 cells formed small tumors with expansive growth properties at the periphery and with signs of rhabdoid differentiation at the center of the tumor. The injection of C212 cells overexpressing cPKCα, β or nPKCε isoforms, when compared to the control ones, generally did not change the major histological characteristics of the tumors. Namely, these tumors maintained the expansive (i.e., non-infiltrative, benign) growth characteristics and histological features of peripheral proliferation and rhabdoid differentiation. In addition, we found only minor differences in the average size of the tumors, the number of dividing cells, and the percentage of Ki67+ (hence proliferating) cells on the histological sections of tumors (these values were somewhat smaller in those tumors which were induced by cells overexpressing cPKCα and β, when compared to the control ones).

However, of great importance, cells overexpressing nPKCδ induced the development of extremely large tumors (often with superficial exulceration and bleeding) which, in few cases, resulted in significant weight loss and eventually death of the animals within the 30-day investigation period. Histologically, these tumors were characterized by markedly high cell division rate, infiltrating (hence malignant) growth properties resulting in destruction of various layers of

different cell types of the skin, and complete lack of rhabdoid differentiation. Therefore, these tumors could be diagnosed as malignant rhabdomyosarcomas. Finally, it was also important to observe that C2C12 myoblasts overexpressing the DN-nPKCδ failed to induce any tumor when injected intradermally to SCID mice. Taken together, these findings also strongly argued for the key role of nPKCδ in promoting *in vivo* proliferation and malignant transformation of C2C12 skeletal muscle cells.

Expression and activity of PKC isozymes alter during chondrogenic differentiation – Role of PKCμ

In parallel with the above experiments performed on various skeletal muscle cells, we also initiated the measurement of the PKC systems in HD mesenchymal chondrogenic cultured. We have shown that these cells express 5 PKC isoforms (cPKCα; nPKCε; aPKCλ and ζ; and PKCμ). We have also found that the expression of the existing isoforms differentially alter during *in vitro* chondrogenic differentiation. Namely, levels of cPKCα, nPKCε, and aPKCλ gradually decreased whereas that of PKCμ increased during culturing (the expression of aPKCζ remained constant). Moreover, we have also shown that the total PKC activity (involving the activities of all existing isoforms) slightly yet significantly decreased in parallel with culturing time. In contrast, the PKCμ specific enzyme activity remarkably increased in the differentiated cultures.

These data suggested the cPKCα, nPKCε, and aPKCλ isoforms may regulate the early whereas PKCμ may participate in the late phase of the differentiation process. To assess this latter hypothesis, we have shown that the PKCμ inhibitor resveratrol significantly altered the morphology of the metachromatic chondrogenic cultures; namely, although it decreased the overall size of the differentiating areas (i.e., it inhibited the chondrogenic differentiation), it induced the development of larger cartilage foci. Finally, we

found that the PKCμ inhibitor inhibited the proliferation of the chondrogenic cultures in a dose-dependent manner. These findings further argued for that PKCμ may play a pivotal role in the positive regulation of proliferation and differentiation of chondrogenic mesenchymal cultures.

DISCUSSION

The nPKCδ plays a central and exclusive role in mediating the mitogenic effect of IGF-I on human skeletal muscle cells

In the first phase of pour experiments, we investigated the effects of IGF-I on in vitro proliferation and differentiation of cultured human skeletal muscle cells and the participation of various signal transduction systems in mediating the effect of the growth factor. In good accord with previous findings we have shown that IGF-I stimulated the proliferation and fusion of human skeletal muscle cells as well as the expression of the differentiation marker. We, however, provided the first evidence that the cellular action of IGF-I is exclusively mediated by PKCδ. This argument was supported by the following data: (1) the effect of IGF-I was completely inhibited by the PKCδ-specific inhibitor Rottlerin but not by the inhibitor of the "conventional" PKCα and γ isoforms or by inhibitors of the MAPK or the PI-3K pathways; (2) IGF-I did not stimulate the phosphorylation of the MAPK Erk-1/2; (3) IGF-I caused the selective translocation of PKCδ, presumably reflecting activation of the isoform

On C2C12 myoblasts, development of cellular action of IGF-I, besides nPKCδ, also involves the nPKCδ-dependent activation of the MAPK pathway

Investigation of the effect of IGF-I-coupled signaling on C2C12 myoblasts revealed similar, yet notably, not identical findings. We have shown that (1) the growth-promoting effect of IGF-I was completely abrogated by the selective inhibition of PKCδ but not by the inhibitors of the cPKCs or the PI-3K system; (2) IGF-I initiated the selective tyrosine phosphorylation of PKCδ, a putative sign of activation; (3) the recombinant overexpression of constitutively active form of PKCδ stimulated cellular growth and mimicked the proliferative action of IGF-I; (4) the recombinant overexpression of kinase inactive form of

PKCδ (DN-PKCδ) inhibited proliferation of C2C12 cells and completely prevented the development of the IGF-I-induced effects. These data – similarly to findings on human muscle – strongly argued for the central involvement of PKCδ in the development of IGF-I-specific mechanism in C2C12 cells as well.

However, on C2C12 myoblasts, we also found that the MAPK pathway is additionally involved in mediating the cellular action of IGF-I. This conclusion was supported by that the effect of IGF-I to promote cellular growth was partially (yet significantly) inhibited by the inhibitor of the MAPK and that IGF-I increased the activity-dependent phosphorylation of the MAPK Erk-1/2. Furthermore, since (1) the inhibition of PKCδ completely whereas that of the MAPK only partially prevented the mitogenic effect of IGF-I; (2) the PKCδ inhibitor Rottlerin effectively inhibited the action of IGF-I to increase phosphorylation of Erk-1/2; (3) the accelerated growth of C2C12 myoblasts overexpressing PKCδ was partially inhibited by the MAPK inhibitor PD098059; our findings also indicated that the involvement of the MAPK system requires the preceding IGF-I-mediated activation of PKCδ, introducing the isoform as an "upstream" regulator of the MAPK pathway.

Certain PKC isoforms play specific, differential, and often antagonistic roles in regulating cellular processes of C2C12 myoblasts

In the second part of our experiments, besides nPKCδ, we investigated the (putative) roles of other PKC isozymes in the regulation of cellular processes of C2C12 myoblasts. We provide the first evidence that certain cPKC and nPKC isozymes play differential and antagonistic roles in regulating the *in vitro* proliferation and differentiation C2C12 myoblasts as well as *in vivo* tumor growth induced by these cells. We have shown that the "conventional" cPKCα and β might act as negative regulators of cellular growth and, moreover, their activities stimulate differentiation of the cells.

The nPKCε has been extensively documented as a key molecule to promote cellular proliferation in various cell types. In the current study, however, we found that this isoform plays an insignificant role in regulating proliferation, differentiation, and the tumor inducing properties of C2C12 myoblast. Since we and other have failed to identify this isoform in C2C12 cells, the lack of effect of recombinant overexpression of the constitutively active nPKCε on cellular growth of the myoblasts is most probably due to the lack of the signaling – substrate system related to this isoenzyme.

The nPKCδ dramatically stimulates the in vitro proliferation of C2C12 skeletal muscle cells and induces in vivo malignant transformation

Our most remarkable data in this investigation were obtained with nPKCδ. This isoform was also very often implicated in the regulation of cellular proliferation and differentiation of numerous cell types. However, in most studies (for example, in human keratinocytes and fibroblast), the isoform was suggested to stimulate differentiation and apoptosis and to inhibit proliferation, whereas, up to date, PKCδ was shown to stimulate proliferation (acting as a prosurvival factor) only in certain breast cancer cell lines.

Therefore, since (1) overexpression of the constitutively active nPKCδ stimulated whereas the kinase inactive DN-nPKCδ mutant inhibited *in vitro* growth of C2C12 myoblasts; (2) overexpression of nPKCδ suppressed the expression of the differentiation marker desmin; (3) the inhibition of PKCδ activity by Rottlerin inhibited cellular proliferation of the control C2C12 cells; (4) nPKCδ overexpresser C2C12 cells, when injected to immunodeficient mice, initiated the development of large and, of great importance, malignantly transformed rhabdomyosarcomas (in contrast to control myoblasts which induced benign tumors of much smaller size); (5) DN-nPKCδ overexpresser myoblasts did not induce tumors in SCID mice; our current findings introduce

nPKCδ as a novel significant player in skeletal muscle biology positively controlling cellular growth. This latter argument is also supported by the above data presenting that nPKCδ plays a central role in mediating the mitogenic effect of IGF-I both in human and C2C12 skeletal muscle cells.

Comparison of the current data with previous experimental findings of our laboratory revealed another intriguing phenomenon. In human epidermal keratinocytes (using similar approaches to those presented in this thesis), we found that the overexpression of cPKCα and nPKCδ stimulated cellular differentiation and inhibited cellular proliferation and tumor growth. Conversely, the activity of cPKCβ and nPKCε increased both *in vitro* and *in vivo* growth of cells and inhibited differentiation. Since (1) our current investigation on C2C12 skeletal muscle myoblasts resulted mostly opposite findings (cPKCβ inhibited growth, nPKCε played minor role in the regulation of proliferation, nPKCδ markedly enhanced cellular and tumor growth), (2) in keratinocytes, the overexpression of the „hyperproliferative" cPKCβ and/or nPKCε did not result in malignant transformation *in vivo* (in contrast to the effect of nPKCδ overexpressed in C2C12 cells); these data strongly suggest that certain PKCs not only isoform-specifically regulate cellular proliferation and differentiation but their effect exert a marked cell-type dependence as well.

PKCμ plays a central role in promoting chondrogenic differentiation

In our experiments, we have also started the investigation of another cell type, the chondrogenic mesenchymal cell cultures. We have found that the characteristic PKC isoform pattern as well as the PKC activity markedly changed as a function of culturing time. Expressions of most of the isozymes (cPKCα, nPKCε, aPKCλ) decreased in parallel with differentiation suggesting that these molecules may play roles in the early phase of the process. The

relatively high level of aPKCζ remained unaltered during culturing which argue for that the isoform may regulate essential biological processes.

We, however, observed opposite findings when measuring PKCμ which, among the investigated cell types, is expressed exclusively in chondrocytes. We have shown that the expression and (even more importantly) activity of PKCμ increased during the differentiation of the cells; i.e., the activity of cPKCα, nPKCε, and aPKCλ was apparently "changed" to that of PKCμ in later phase of the process. Since functional studies moreover revealed that inhibition of PKCμ significantly suppressed proliferation and differentiation (hence cartilage formation), our results strongly suggest the pivotal role of PKCμ in the positive regulation of (late processes of) chondrogenic differentiation.

Potential application of the experimental findings

Our experimental findings presented in the current thesis may have relevant impact for applied research, for biotechnology-related research and development, and (hopefully) for clinical practice. Recently, in numerous clinical trials, the investigators intend to suppress the accelerated growth of various tumors by changing the activity of the PKC isoforms. Hence, our data that certain PKC isozymes specifically regulate *in vitro* and *in vivo* cellular proliferation may facilitate the proper planning and execution of such studies as well as the analysis of biologically and therapeutically effect pharmacological agents. For example, our experimental findings suggest that, in malignant skeletal muscle tumors, the selective inhibition of nPKCδ-specific activity (which induces hyperproliferative transformation and mediates the mitogenic action of IGF-I on myoblasts) may serve as a fine therapeutic strategy. Alternatively, a complementary approach could be the application of such molecules which selectively stimulate the activity of cPKCα and β which inhibit proliferation and promote differentiation. Finally, in degenerative diseases of the

cartilage, the selective stimulation of PKCμ (and hence the process of chondrogenesis) may also have a therapeutic impact.

Our experimental data, however, also implicate a potential danger of the application of PKC-acting agents. As we have shown above, the growth-promoting or -inhibiting role of a given PKC isoform is strongly dependent of the actual cell type. The nPKCδ, for example, was almost exclusively described as a negative regulator of cellular proliferation. As a marked contrast, however, in skeletal muscle cells we have proven that this isoform rather stimulates proliferation (and not differentiation) and that its constitutive presence results in malignant transformation. Hence, if one systemically increases the activity of nPKCδ (e.g., in the treatment of skin malignancies), it should be taken into account that such an intervention (besides suppressing the unwanted growth of the targeted tumor) may induce malignant transformation in another cell type (e.g., in skeletal muscle) by acting on the very same PKC isozyme.

SUMMARY

In our studies we investigated the participation of the protein kinase C (PKC) isoenzyme family and of other signaling systems in the regulation of *in vitro* and *in vivo* proliferation and differentiation of skeletal muscle cells and chondrocytes. We found that the nPKCδ isoform plays an exclusive role in the development of the mitogenic effect (i.e. to promote growth and differentiation) of IGF-I, a key molecule of human skeletal muscle regeneration. However, on mouse C2C12 myoblasts, we have also shown that besides the central involvement of nPKCδ-specific activity, the MAPK pathway also participates in mediating the effect of IGF-I. In addition, it was also proven on these cells that the nPKCδ functions as an "upstream" regulator of the MAPK pathway; i.e. its preceding activation is required for the stimulation of the MAPK system. Furthermore, we also found that stable recombinant overexpression of various PKC isoforms in C2C12 myoblasts differentially affected the functional and morphological features of the cells. The overexpression of cPKCα and β decreased the growth rate of the cells whereas that of nPKCε did not exert any effect. As a marked contrast, constitutive overexpression of nPKCδ dramatically stimulated *in vitro* cellular proliferation, suppressed the expression of the differentiation marker desmin, and promoted the *in vivo* development of large, malignantly transformed tumors in immunodeficient mice. Finally, using chondrogenic high-density chicken limb bud mesenchymal cultures, we have shown that the unique PKCμ plays a central role in the regulation of the (late) events of chondrogenic differentiation. These findings strongly argue for the specific yet often antagonistic functions of certain PKC isoforms in the regulation of growth and differentiation of skeletal muscle cells and chondrocytes.

PUBLICATIONS

The thesis was built on the following in extenso scientific publications:

1) ***Czifra G.****, Tóth I.B., Marincsák R., Juhász I., Kovács I., Ács P., Kovács L., Blumberg P.M., and Bíró T.* (2006): Insulin-like growth factor-I-coupled mitogenic signaling in primary cultured human skeletal muscle cells and in C2C12 myoblasts. A central role of protein kinase Cδ. *Cell. Signal.* ***Epub.*** **IF: 4.741**

2) ***Czifra G.****, Szöllősi A., Juhász I., Kiss A., Erdődi F., Varga A., Kovács I., Ács P., Kovács L., Blumberg P.M., and Bíró T.* (2006): Protein kinase C isoforms differentially regulate proliferation, differentiation, and tumorigenicity of skeletal muscle cells. *Exp. Cell. Res.* (submitted for publication)

3) *Zákány R., Szűcs K., Bakó É., Felszeghy Sz.,* ***Czifra G****., Bíró T., Módis L., and Gergely P.* (2002): Protein phosphatase 2A is involved in the regulation of protein kinase A signaling pathway during in vitro chondrogenesis. *Exp. Cell. Res.* **275**:1-8. **IF: 4.007**

Additional in extenso scientific publications:

1) *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: 2.503**

2) *Papp H.,* ***Czifra G.,*** *Lázár J., Boczán J., Gönczi M., Csernoch L., Kovács L., and Bíró T.* (2003): Protein kinase C isozymes regulate proliferation and high cell density-mediated differentiation of HaCaT keratinocytes. *Exp. Dermatol.* **12**:811-824. **IF: 1.707**

3) *Varga E., Nagy N., Lázár J,* ***Czifra G****., Bak I., Bíró T., and Tósaki Á.* (2004): Inhibition of ischemia/reperfusion-induced damage by dexamethasone in isolated working rat hearts: the role of cytochrome c release. *Life Sci.* **75**:2411-2423. **IF: 2.158**

4) *Varga A.,* ***Czifra G****., Tállai B., Németh T., Kovács I., Kovács L., and Bíró T.* (2004): Tumor grade-dependent alterations in the protein kinase C isoform pattern in urinary bladder carcinomas. *Eur Urol.* **46**:462-465. **IF: 2.651**

5) *Papp H.,* ***Czifra G.,*** *Bodó E., Lázár J., Kovács I., Aleksza M., Juhász I., Ács P., Sipka S., Kovács L., Blumberg P.M., and Biró T.* (2004): 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: 4.812**

6) *Zákány R., Szijgyártó Z., Matta Cs., Juhász T., Csortos Cs., Szűcs K.,* ***Czifra G.****, Bíró T., Módis L., and Gergely P*. (2005): Hydrogen peroxide inhibits formation of cartilage in chicken micromass cultures and decreases the activity of calcineurin: implication of ERK1/2 and Sox9 pathways**.** *Exp Cell Res*. **305**:190-199. **IF: 4.007**

7) *Kovács I., Pocsai K.,* ***Czifra G.****, Sarkadi L., Szűcs G., Nemes Z., and Rusznák Z.* (2005): TASK-3 immunoreactivity shows differential distribution in the human gastrointestinal tract. *Virchows Arch.* **446**:402-410. **IF: 2.227**

8) *Bodó E., Bíró T., Telek A.,* ***Czifra G****., Griger Z., Tóth I.B., Mescalchin A., Ito T., Bettermann A., Kovács L. and Paus R.* (2005): A "hot" new twist to hair biology – involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *Am. J. Pathol.* **166**:985-998. **IF: 6.441**

9) *Szabó G., Szentandrássy N., Biró T., Tóth I.B.,* ***Czifra G.****, Magyar J., Bányász T., Varró A., Kovács L., and Nánási PP*. (2005): Asymmetrical distribution of ion channels in canine and human left-ventricular wall: epicardium versus midmyocardium. *Pflügers Arch.* **450**:307-316. **IF: 2.260**

Cumulated impact factor of the above publications (based on JCR 2004): 37,514

Abstracts in scientific journals:

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) *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 to capsaicin. *Acta Physiol. Hung.* **89**:13

5) *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**:348.

***6) 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**:278.

***7)** 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. No. 195

***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. Dermatol. Sci.* **32(2):169.** No. 195

***9) Czifra G.**, Lázár J., Kern A., Juhász I., Kovács I., Kovács L., Blumberg P.M., and Bíró T.* (2003): PKCδ mediates the growth promoting effect of IGF-I on cultured human skeletal muscle cells. *Neuromuscular Disord.* **13(7-8)**:621-622.

***10)** Bodó E., Géczy T., Lázár J., Kovács I., **Czifra G.**, Bettermann A., Kovács L., Paus R., and Bíró T.* (2003)*:* The cutaneous vanilloid receptor 1 expression suggests multiple functions beyond sensory nerve signaling. *J. Invest. Dermatol.* **121(1)**:218. No. 823

***11)** Bodó E., Géczy T., Lázár J., Kovács I., **Czifra G.,** Bettermann A., Kovács L., Paus R., and Bíró T.* (2003): The cutaneous vanilloid receptor 1 expression suggests multiple functions beyond sensory nerve signaling. *J. Dermatol. Sci.* **32**:169. No. 823

***12)** 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

***13)** Szegedi A., **Czifra G.**, Schmidt E., Hunyadi J., and Bíró T.* (2004): Protein kinase C isoforms have different effect on the expression of desmoglein 1, 3 and P-cadherin in HaCaT keratinocytes. *J. Invest. Dermatol.* **123(2)**:011.

***14)** Bodó E., Bíró T., Telek A., **Czifra G.**, Griger Z., Tóth I.B., Lázár J., Mescalchin A., Ito T., Bettermann A., Pertile P., Kovács L., and Paus R.* (2004): A "hot twist" to hair biology – Involvement of vanilloid receptor-1 (VR1) signalling in human hair growth control. *Exp. Dermatol.* **13(9)**:581.

***15)** Matta C., Juhász T., Szijgyártó Z., Szűcs K., **Czifra G.**, Módis L., Gergely P., Zákány R* (2004): Protein kinase C isoenzymes regulates chondrogenesis of mesenchymes. *Tissue Antigens* **64(4)**:435-435.

***16)** Juhász T., Szijgyártó Z., Matta C., Szűcs K., **Czifra G.**, Bakó É., Zákány R., Módis L., Gergely P.* (2004): The role of calcineurin in the inhibitory

effect of oxidative stress on cartilage formation *Tissue Antigens* **64(4)**:435-436.

17) *Tóth I.B., Géczy T.,* ***Czifra G.****, Seltmann H., Paus R., Kovács L., Zouboulis C.C., Bíró T.* (2005)*:* The vanilloid receptor 1 (TRPV1) is expressed and functionally active on human SZ95 sebocytes. *J. Invest Dermatol.* **124(4)**:A68-A68. 405 Suppl. S

18) *Juhász T., Matta C., Szijgyártó Z.,* ***Czifra G.****, Bíró T., Gergely P., Módis L., Zákány R.* (2005)*:* Calcineurin regulates chondrogenesis via the modulation of ERK1/2 activity. *FEBS J.* **272**:307-307. Suppl. 1

19) *Matta C., Juhász T., Szijgyártó Z.,* ***Czifra G.****, Bíró T., Gergely P., Módis L., Zákány R.* (2005)*:* The role of protein kinase C isoenzymes chondrogenesis of micro-mass cell cultures. *FEBS J.* **272**:310-310. Suppl. 1

20) *Rusznák Z., Kovács I., Pocsai K.,* ***Czifra G.****, Sarkadi L., Nemes Z., Szűcs G* (2005): Distribution of TASK-3 channels in healthy and pathological human tissue samples *FASEB J.* **19(5)**:A1160-A1160. Part 2 Suppl. S