Ph.D. THESIS

Role of calcineurin in the *in vitro* regulation of cartilage differentiation and in the signal transduction pathways of human mononuclear cells

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2007

INTRODUCTION

Calcineurin

Calcineurin (PP2B) is a binuclear [Fe^{2+}-Zn^{2+}] metalloenzyme, a phospho-Ser/Thr-specific protein phosphatase consisting of a 59 kDa catalytic subunit (calcineurin A) and a smaller 19 kDa Ca^{2+}-binding regulatory subunit (calcineurin B). Calcineurin can modulate the cellular function directly, by physically interacting with and dephosphorylating relevant target proteins, or indirectly, by activating upstream signalling pathways (e.g. NFAT, NF-κB, JNK, CREB) that control the expression level of specific proteins or their biological activity.

Calcineurin and NFAT (nuclear factor of activated T-cell) are crucial in the mesenchymal cell differentiation and in the signalling of immune cells. Up to, there has not been direct evidence for the positive role of calcineurin in the cartilage differentiation. The classical mechanism by which calcineurin is regulated *in vivo* is via changes in the intracellular Ca^{2+}. The elevated intracellular calcium can induce chondrogenesis through a calcineurin/NFAT signalling axis that activates BMP (bone morphogenic proteins) expression. Calcineurin also plays essential roles in T-cell receptor-mediated peripheral T-cell activation, cell proliferation, and death. Calcineurin promotes the nuclear translocation of NFAT4 by dephosphorylating the transcription factor. NFAT4 takes a prominent part in the transcription of several genes of pro-inflammatory cytokines, cell surface molecules, and Fas ligand, too. We reported a significant decrease of calcineurin activity in peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE). It was demonstrated also that the

glucocorticosteroid-treatment of PBMC from healthy controls exhibited lower calcineurin activity in the presence of Ca-ionophore and phorbol ester. Our observation suggested that protein kinase (PKC) isoenzymes might have some role in the regulation of calcineurin in T-cells.

Cartilage differentiation

The differentiation of mesenchymal cells into chondrocytes is a multistep pathway including the recruitment of mesenchymal progenitor cells, the subsequent condensation of these cells, followed by the differentiation of the condensed mesenchymal cells into chondrocytes. At this stage the cells surround themselves with an abundant layer of extracellular matrix and start to express cartilage-specific extracellular matrix molecules (e.g. collagen type II, IX, and XI, linker protein, and aggrecan). Expression of type II collagen and core protein of aggrecan is regulated by the transcription factor Sox9, which is the product of the supposed master gene of cartilage differentiation. Several Ser/Thr-specific protein kinases (e.g. MAPK/Erk1/2/p38, PKC, PKA) and phospho-Ser/Thr-specific protein phosphatases (e.g. PP2A, PP2B) play roles in the cartilage differentiation, which influence the activity of different transcription factors (e.g. CREB, Sox9), by this means, they regulate the transcription of cartilage-specific genes (e.g. aggrecan, collagen, N-cadherin, fibronectin, integrin). Activity of Sox9 protein is regulated by phosphorylation of a serine 211 residue which is a target of cAMP-dependent protein kinase (PKA). Our group has given the first evidence of the possible involvement of one of the major cellular phosphatases, PP2A, in the regulation of PKA/CREB axis during chondrogenesis.

Mitogen-activated protein kinases

Recent studies have indicated that the inhibitors of calcineurin, both CSA and FK506, also inhibit members of mitogen-activated protein kinases (MAPKs) pathway in T lymphocytes. The MAPKs are responsible for the conversion of a large number of extracellular stimuli into specific cellular responses that range from positive and negative roles in cell proliferation, differentiation and apoptosis to regulation of inflammatory and stress responses. The MAPK/Erk1/2 is widely accepted as one of the major mediator of mitogenic extracellular stimuli and also plays a role in the proliferation, differentiation of several cell types. There are accumulating data on the role of calcineurin in the regulation of the activity of MAPKs.

The oxidative stress

The reactive oxygen species (ROS) and the hydrogen-peroxide (H_2O_2) are affect several different signal transduction pathways, activate diverse enzymes of signalling cascade, and also play roles in the regulation of differentiation and various cell functions in articular tissues. Although calcineurin is declared as one of the major mediator of the signals elevating intracellular Ca^{2+} level, but recently a further mechanism for regulating calcineurin has been discovered, which involves redox reactions of Fe^{2+} ion in the active site of the enzyme. The activity of calcineurin is affected by extracellular oxidants, in particular H_2O_2. During inflammatory diseases of articular cartilage a number of reactive oxygen species are liberated, which can influence of the enzyme activity of calcineurin probably due to a bridging of two closely spaced Cys residues.

Protein kinase C

The enzymes of the PKC superfamily phosphorylate Ser and Thr residues in many target proteins and play central roles in the regulation of various cellular processes in numerous cell types. PKC isoenzymes can be classified into three major groups that differ in their cofactor requirements. These are known as the conventional PKC (cPKC: α, βI, βII, and γ), the novel PKC (nPKC: δ, ε, η, and θ), the calcium and phorbol-ester-independent atypical PKC (aPKC: ζ,, and λ/ι,) isoforms, and the unique PKCμ. Both cPKCs and nPKCs can be activated with phorbol ester, however, the most important difference is that nPKCs are insensitive for Ca^{2+} signalling. These isoforms, possessing characteristic tissue and cellular distribution, regulate in an isoform-specific manner various cellular functions such as proliferation, differentiation, cytokine production and release, and receptor-mediated signal transduction.

PKC and Ca^{2+}/calmodulin dependent protein kinase II (CaM-kinase II) are able to phosphorylate the catalytic subunit of calcineurin. The phosphorylation of the enzyme by PKC does not influence its activity while the phosphorylation of the catalytic subunit of calcineurin by CaM-kinase II inhibits the enzyme. Recently, growing number of endogenous calcineurin-binding proteins have also been discovered affecting the enzyme activity. They could be classified as dual regulators, anchoring proteins and inhibitors of calcineurin. *Ca*lcineurin *bin*ding protein (Cabin 1) belongs to the group of endogenous inhibitors of calcineurin, which results in the inhibition of the enzyme activity by binding to the calcineurin. Cabin 1 is hypophosphorylated in non-activated T-cellsand in response to PKC activation it becomes hyperphosphorylated exhibiting higher affinity for calcineurin.

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease. In SLE various abnormalities affecting signal transduction pathways have been reported, for example abnormal NF-κB (nuclear factor-κB) activity and intracellular distribution of NFAT1, overproduction of pro-inflammatory cytokines, decreased expression of TCR zeta chain and PKCθ, decreased PKC dependent protein phosphorylation. Previously we have shown a significant decrease of the calcineurin activity in healthy and lupus peripheral blood mononuclear T-cellstreated with Ca-ionophore and PMA in the presence or the absence of glucocorticosteroid mostly used for the treatment of SLE in the active period of the disease. Our observation suggested that the PKC enzymes might have some role in the regulation of calcineurin in T-cells.

AIMS

The reactive oxygen species can influence the possible renewal of damaged cartilage. To elucidate this we investigated the effects of oxidative stress on the formation of cartilage in high density micromass cultures of embryonic chondrogenic cells of chicken. Our aims were

- to study the role of calcineurin in chondrifying mesenchymal cells of chicken,
- to investigate the phosphorylation and dephosphorylation processes during the cartilage differentiation,
- to explore the effect of oxidative stress.

We also studied the possible role of PKC isoenzymes upon stimulation by phorbol-ester and Ca-ionophore in the inhibition of calcineurin in PBMC from healthy controls. We aimed at investigating

- the possible role of PKC isoenzymes upon stimulation by phorbol-ester and Ca-ionophore in the inhibition of calcineurin in PBMC from healthy controls,
- a target molecule regulated by PKC which would have a negative role in the calcineurin activity of the mononuclear cells stimulated with Ca-ionophore and/or PMA.

MATERIALS AND METHODS

Cell culturing

Distal parts of the limb buds of Ross hybrid chicken embryos of Hamburger-Hamilton stages 22-24 were removed and primary micromass cultures of chondrifying mesenchymal cells were established from cell suspensions with a density of 1.5 x 10^7 cell/ml. Colonies were nourished with Ham′s F12 medium, supplemented with 10 % fetal calf serum and were kept at 37°C in the presence of 5 % CO_2 and 95 % humidity in a CO_2 incubator.

Light microscopical morphology with image analysis

High-density cultures established from 30 µl droplets of chondrogenic limb bud mesenchymal cells, of different experimental groups cultured on the surface of round coverglasses placed into wells of 24-well culture plates, were fixed in a 4:1 mixture of absolute ethanol and 40 % formaldehyde at day 6 of culturing. Cultures were stained with 0.1 % dimethylmethylene blue dissolved in 3 % acetic acid, washed in acetic acid. Some cultures were stained with 0.1 % aqueous DMMB. The size of the metachromatic cartilaginous areas was measured in 10 cultures of each experimental group in 4 independent experiments by computer-assisted image analysis.

Investigation of cell proliferation with [^{3}H]-thymidine labelling

15 µl droplets of cell suspension were inoculated into wells of 96-well microtiter plates. Medium containing 1 µCi/ml [^{3}H]-thymidine was added to the wells for 16 hours on days 2, 3 or 6. After washing with PBS,

proteins were precipitated with ice-cold 5 % trichloroacetic acid, washed with PBS again, digested with 0.25 % trypsin dissolved in calcium magnesium free-phosphate buffered saline and harvested with a semiautomatic cell-harvester. Harvested colonies were dried on scintillation filter paper and radioactivity was counted by a liquid scintillation counter. Measurements were carried out in 10 samples of each experimental group in 4 independent experiments. Data were statistically analysed with F-test.

Investigation of cellular viability

For investigation of cellular viability, cartilage cells cultured were treated with 10 µl MTT reagent and incubated for 2 hours at 37°C. Following addition of 100 µl of MTT solubilizing solution to the samples the absorption was measured at 570 nm. After the stimulation of PBMC, 2 x 10^6 cells/100 µl were treated with alamarBlue according to the manufacturer's instruction. The cells were incubated in a CO_2 incubator at 37°C for 45 min. The fluorescence of the alamarBlue was determined at 530 nm excitation and at 590 nm emission. The stimulating agents of PKC had no significant effects on the arbitrary fluorescence unit (AFU) measured in the samples.

Treatments of cartilage cells

Cell cultures were treated on days 2 and 3 with 0.1, 1.0 or 4.0 mM H_2O_2 for 30-30 minutes. Activity of calcineurin was inhibited with the application of 2 µM CsA on culturing days 2 and 3 for 4-4 hours. Erk1/2 was inhibited with continuous application of 5 µM PD098059 started on day 1.

Preparation of human peripheral blood mononuclear cells and characterizations of cells by flow cytometry

PBMC, containing 88-95 % lymphocytes and 5-12 % monocytes, were prepared from the heparinised blood of healthy donors. The averages of various cellular subsets were detected by flow cytometry: CD3+ 69.4 %, CD19+ 11.5 %, CD56+ 0.8 %, CD14+ 8.3 %.

Stimulation of PBMC

The cells (5 x 10^6 cells/ml) were incubated with 5 μM of Ca-ionophore (A23187) or/and 80 nM of phorbol 12-myristate 13-acetate (PMA) for 4 hours in a CO_2 incubator at 37°C. For PKC inhibition studies the cells were preincubated for 1 hour with various cell-permeable inhibitors (1 μM GF109203X, 200 nM Gö6976, 10 μM Rottlerin) prior the treatment with Ca-ionophore or/and PMA.

Preparation of cell extracts

Cell cultures were harvested after the treatments or immediately after the oxidative stress for cartilage cells, on respective days of culturing. Cell pellets were suspended in 100 μl of homogenization buffer. After storing them at -70 °C, suspensions were sonicated and centrifugated, the supernatants were used in prompt for calcineurin assays. For Western blotting total cell lysates were used. For RT-PCR analysis cartilage colonies were washed by using RNAses free physiologically sodium chloride then the cultures were stored frozen at -70 °C.

Assay of calcineurin

Calcineurin activity was measured by the release of $^{32}P_i$ from ^{32}P-labelled protein phosphatase inhibitor-1 (780 cpm/pmol). The assay mixture (30 μl) containing protease inhibitors, 40 μg/ml calmodulin, 0.2 mM $CaCl_2$, 100 nM okadaic acid, 2 nM protein phosphatase inhibitor-2, an appropriate amount of extract (2-3 mg/ml protein), and ^{32}P-labelled protein phosphatase inhibitor-1 (20-30000 cpm/reaction mixture) was incubated at 30°C for 20 minutes. The reaction was terminated by addition trichloroacetic acid and bovine serum albumin. After centrifugation $^{32}P_i$ of 180 μl of the supernatant fraction was determined by Cerenkov counting in a liquid scintillation counter.

Immunoprecipitation of Cabin 1

After sonication of cell suspensions, the samples were centrifuged and the supernatants were used for immunoprecipitation analysis. Cell lysates containing 200 μg of protein were incubated with 0.25 μg/ml anti-rabbit IgG antibody and Protein A Sepharose for 2 h at 4°C. After centrifugation the supernatants were incubated with 5 μl anti-Cabin 1 antibody for 2 h at 4°C. Then 50 μl Protein A Sepharose beads were added to the precleared samples containing antibody-protein complexes and they were incubated overnight at 4°C. After collecting the antigen-antibody-protein A complexes by centrifugation, pellets were washed three times with immunoprecipitation buffer. For SDS-PAGE, antigen-antibody-protein A samples were prepared by adding electrophoresis sample buffer and boiling.

Western blot analysis

Total cell lysates were examined by Western blot. Samples for SDS-PAGE were prepared by adding electrophoresis sample buffer to cell lysates and boiled. For cartilage cells about 70-80 μg of protein was separated by SDS-PAGE gel. For mononuclear cells 10-50 μg of protein was used. Proteins were transferred electrophoretically to a nitrocellulose membrane. After blocking, the membranes were washed and exposed to the properly diluted primary antibodies overnight at 4°C. Then the membranes were incubated with the second antibodies for 2 hours. The signals were detected by enhanced chemiluminescence.

RT-PCR Analysis

Total RNA was isolated from cells. We used 2 μg RNA for reverse transcriptase reaction. The primer pairs for polymerase chain reaction were designed. Amplifications were performed in a thermocycler as follows: 94°C, 1 min, followed by 30 cycles (94°C, 30 sec, 54°C, 30 sec, 72°C, 30 sec) and then 72°C, 5 min. PCR products were analysed by electrophoresis in 1.2 % agarose gel containing ethidium bromide.

Quantitative real time RT-PCR

For two steps quantitative real time RT-PCR based TaqMan probe the primers and probes of GAPDH, aggrecan, Sox9 and calcineurin were designed by ABI. The reaction mixture of PCR contained 250 ng or 500 ng cDNA. The PCR were performed as follows: 95°C, 10 min, followed by 45 cycles (95°C, 25 sec, 60°C, 1 min). The software collects the fluorescence signal at the last step (60°C, 1 min). The internal reference dye was ROX and the reference gene was GAPDH during the assay. To

determine the change of mRNA level the software used the following algorithm.

Change compared to the mRNA level of the studied gene in the untreated sample = $2^{\Delta\Delta C}{}_{T}$, where $\Delta\Delta C_T = \Delta C_{T\ (control)} - \Delta C_{T\ (treated)}$,

$\Delta C_{T\ (control)}$ = average $C_{T\ (untreated\ sample,\ studied\ gene)}$ − average $C_{T\ (untreated\ sample,\ GAPDH)}$

$\Delta C_{T\ (treated)}$ = average $C_{T\ (treated\ sample,\ studied\ gene)}$ − average $C_{T\ (treated\ sample,\ GAPDH)}$

RESULTS

The change of the cartilage specific markers and Sox9 transcription factor during the cartilage differentiation

The differentiation of mesenchymal cells into cartilage cells is highly regulated by several transcription factors, e.g. Sox9. We observed that the expression of Sox9 regulated the transcription of aggrecan was dominant at the beginning of chondrogenesis. Both the mRNA and the protein level of Sox9 were the highest at days 2-4. The mRNA level of aggrecan was increased during the differentiation. The changes of mRNAs were studied by using the conventional and the relative quantitative RT-PCR.

The effect of oxidative stress on the chondrogenesis

In micromass cultures of mesenchymal cells derived from chick limb buds a spontaneous cartilage formation occurs in 6 days. Relative efficiency of cartilage formation can be estimated with the analysis of metachromasia, detecting the amount of glycosaminoglycan side chains of proteoglycans of cartilage matrix with dimethylmethylene blue staining. When cartilage formation was disturbed by oxidative stress induced with different concentrations of hydrogen peroxide applied on days 2 and 3 of culturing, a significant and concentration dependent decrease of metachromasia was visible in 6-day-old cultures.

A H_2O_2-concentration dependent decrease of both mRNA levels of aggrecan and Sox9 was demonstrated by RT-PCR and quantitative RT-PCR. Although proliferation rate of cartilage cells was reduced significantly even with the use of 0.1 mM of H_2O_2 (from 100 % to 75 %),

but the viability of chondrocytes remained around 80 % compared to the untreated controls. For further experiments we have chosen 1 mM of H_2O_2, because it significantly inhibited chondrogenesis without any apparent cytotoxic effect.

The role of calcineurin in the chondrogenesis

First we demonstrated the presence and catalytic activity of calcineurin in micromass cultures. Calcineurin was detectable in chicken cartilage cells throughout the culturing period and expression of the mRNA of calcineurin gradually decreased as revealed by RT-PCR technique. According to Western blot analyses these changes were less characteristic at protein level. Since the presence of an enzyme in a cell does not mean necessarily its active function, the activity of calcineurin in cell homogenates of cartilage cultures of different ages were also assayed. The activity of calcineurin significantly decreased by day 6 of culturing and was the highest on days 1 and 2.

To investigate the role of calcineurin in the cartilage differentiation we applied 2 μM CsA, a specific inhibitor of calcineurin, on the chondrogenic days 2 and 3 for 4-4 hours. It caused a sever reduction in cartilage formation as it was detected with metachromatic staining of 6-day-old HDCs. CsA also lowered the levels of the mRNAs of aggrecan and Sox9. mRNA and protein expression of calcineurin catalytic subunit increased after the treatment, probably a compensatory elevation due to the direct inhibition by CsA. CsA inhibited the activity of calcineurin about 30 % assayed in cell lysates.

Exposure of HDCs to 1 mM H_2O_2 for 30-30 min on days 2 and 3 of culturing resulted in the inhibition of calcineurin activity with 40 %.

H_2O_2 also reduced both mRNA and protein levels of calcineurin as revealed by RT-PCRs and Western blots. These data were recorded from cell extracts of 3-day-old HDCs prepared immediately after the second H_2O_2 treatment.

The role of MAPK/Erk1/2 in the chondrogenesis

As MAPK pathways are supposed to be mediators of oxidative stress and Erk1/2 is a negative regulator of chondrogenesis. We studied the effect of PD098059, an inhibitor of Erk1/2, on the formation of cartilage. Pre-treatment of HDCs with PD98059 stimulated the cartilage matrix deposition and decreased the depletion of cartilage matrix in the presence of H_2O_2 or CsA based on metachromatic analyses.

We also investigated the activity of Erk1/2 in micromass cultures exposed to H_2O_2 treatments. Since the activity of Erk1/2 is regulated by phosphorylation, the change of phosphorylation level of this kinase was detected with an antibody raised against the dual phosphorylated variant of Erk1/2. An extremely elevated level of phosphorylation of Erk1/2 both on Thr and Tyr was found in 3-day-old cells following the repeated H_2O_2 treatments. Furthermore, inhibition of calcineurin with CsA also elevated the amount of the dual phosphorylated Erk1/2.

PD98059 reduced the activity of Erk1/2 both in CsA and H_2O_2-treated HDCs, and also prevented, at least partly, the decrease of the expression of mRNAs of either Sox9 or the core protein of aggrecan. We have detected an increase of the phosphorylation state of Sox9 in 3-day-old HDCs exposed to H_2O_2 or CsA, while inhibition of Erk1/2 activity with PD98059 resulted in a severe decrease of the phosphorylation of the transcription factor.

The treatment of cartilage cells with PD098059 increased the transcription of calcineurin but had no effect on the protein level. The inhibition of Erk1/2 elevated both mRNA and protein levels of calcineurin in cell cultures exposed to oxidative stress.

We also studied the effect of the inhibition of MEK/Erk1/2 pathway on the calcineurin activity. PD098059 caused a significant increase of the activity compared to the untreated control. There was no considerable change of calcineurin activity in the cartilage cells treated with CsA or H_2O_2 after the application of PD098059.

NFAT4 transcription factor is one of the main transducer of the activation of calcineurin and is proposed to stimulate chondrogenesis via BMPs. In our experimental system we found only a slight modulation in the phosphorylation level of NFAT4 under any investigated conditions.

Effect of various protein kinase C inhibitors on the calcineurin activity of human PBMC stimulated by phorbol-ester and Ca-ionophore

PKC inhibitors were used as follows: GF109203X, an inhibitor of the classic and the novel, Gö6976, an inhibitor of the classic, and Rottlerin, an inhibitor of the δ type of PKC isoenzymes. None of the three PKC inhibitors were found to have any significant effect on the basic activity of calcineurin in the absence of stimulating agents. Stimulation of cells with PMA resulted in a significant decrease of calcineurin activity as compared to the non-stimulated control (68 % versus 100 %). Similarly to PMA, Ca-ionophore also resulted in a significant decrease in the activity of calcineurin (58 % versus 100 %). Moreover, it was a slightly higher reduction than that observed in the presence of PMA. There was no

significant change in the calcineurin activity of PBMC simultaneously treated with both activating agents as compared to the enzyme activity of PBMC stimulated with Ca-ionophore alone.

PKC inhibitors, GF109203X and Gö6976, reversed the phosphatase activity in the stimulated cells approximately with the same efficacy (in the presence of PMA: 85 % and 88 % vs. 68 %, in the presence of Ca-ionophore: 87 % and 79 % vs. 58 %, in the presence of both activating agents: 92 % and 90 % vs. 47 %) while Rottlerin was without effect under these conditions. It is known that GF109203X is a less selective inhibitor of PKC isoenzymes than Gö6976. Gö6976, a selective inhibitor of cPKC isoenzymes, alone was able to counteract the inhibition of calcineurin suggesting the involvement of cPKC α, β, γ isoenzymes in the PMA and/or Ca-ionophore induced changes.

The RT-PCR and Western blot analyses have shown that neither PMA nor Ca-ionophore applied alone modified the mRNA and protein levels of the enzyme, which imply that the decrease in the calcineurin activity of PBMC treated with PMA and Ca-ionophore is not due to the inhibition of transcription or translation of calcineurin.

Cabin 1 is a possible signal transducer between PKC and calcineurin

As the phosphorylation of calcineurin by PKC does not affect its enzymatic activity, one can suppose that an upstream signal transduction molecule activated by PKC could be involved in the reduction of calcineurin activity. It has been reported that in response to PKC activation Cabin 1 becomes hyperphosphorylated exhibiting a higher affinity for calcineurin and the binding of Cabin 1 to the enzyme inhibits its activity.

To investigate the possible signal transducer role of Cabin 1 in this signalling pathway, Cabin 1 was immunoprecipitated with a polyclonal antibody. After immunoprecipitation the phosphorylation state of Cabin 1 on Ser residues were analyzed by immunoblotting. The phosphorylation state of Cabin 1 was remarkably enhanced by activating agents of PKC applied alone or in combination. On the other hand, PKC inhibitors (GF109203X and Gö6976) significantly reduced the hyperphosphorylation of Cabin 1. Rottlerin had no effect on the hyperphosphorylation of Cabin 1 as compared to the data of stimulated PBMC.

DISCUSSION

Protein phosphatases play equally important role as protein kinases in the regulation of protein phosphorylation processes of different signal transduction pathways controlling cell and tissue differentiation. Oxidative stress is one of the factors among the harmful effects acting during inflammatory diseases and triggering loss of chondrocytes. It is known that activity of calcineurin is modulated by reactive oxygen species.

We have shown the presence and active function of calcineurin in micromass cultures. We propose a positive role of calcineurin in the regulation of *in vitro* chondrogenesis of chicken micromass cultures, since we have demonstrated an inhibitory effect of the calcineurin inhibitor CsA in cartilage formation of HDCs and in the mRNA level of aggrecan and Sox9. Calcineurin was inhibited by oxidative stress and oxidative stress decreased the formation of cartilage in HDCs further supporting the positive role of calcineurin in the regulation of chondrogenesis. Although calcineurin was proposed to be involved in the regulation of chondrogenesis via NFAT4 and BMP, we failed to find any significant changes in the phosphorylation status of NFAT4 either in hydrogen peroxide or CsA-treated HDCs. This result raises the possibility of the involvement of other target molecules of calcineurin and/or oxidative stress in our experimental system.

We observed an increased cartilage formation in the presence of the MEK inhibitor PD98059, while the phosphorylation of the chondrogenic transcription factor Sox9 was decreased. Since phospho-Sox9 initiates and stimulates the transcriptional activity of genes of cartilage matrix-specific macromolecules, like type II collagen and core

protein of aggrecan, we assume that the chondrogenesis promoting effect of the inhibition of Erk1/2 with PD98059 influences other downstream targets facilitating cartilage formation. It is to be noted that our data do not rule out the possibility that Erk1/2 itself may phosphorylate Sox9. Interestingly phosphorylation of Sox9 was also elevated following H_2O_2 or CsA treatments, but cartilage formation was reduced in both cases. This contradiction can be explained with the presence of other downstream targets of oxidative stress inhibiting chondrogenesis and with the possible role of calcineurin in the dephosphorylation of Sox9.

Moreover, we found an elevated activity of Erk1/2 in H_2O_2 and in CsA-treated HDCs and the inhibition of Erk1/2 with PD98059 had a partial protecting effect either on H_2O_2 or CsA induced impairment of chondrogenesis. Additionally, phosphorylation of Sox9 remained higher in the treated samples than in the control ones. These findings further support our assumption that increased activity of Erk1/2 can be the reason of the reduced cartilage formation following oxidative stress and calcineurin may exert a negative effect either on Erk1/2 and/or Sox9 pathways during chondrogenesis.

The inconsistency of our results describing elevated Sox9 phosphorylation parallel to inhibited cartilage formation either after oxidative stress or CsA treatments can be explained by the elevated activity of cAMP-dependent protein kinase (PKA) in oxidatively stressed or CsA treated cartilage cells. PKA is known as a positive regulator of chondrogenesis in HDCs, and also stimulates the transcriptional activity of Sox9. Furthermore, calcineurin and PKA have a common anchoring protein (AKAP79) targeting them together to the respective subcellular compartments, which suggests a cooperation of the two enzymes in

different intracellular processes. It also implicates the possibility of the regulatory effect of the two enzymes on each other, since both of them could be regulated by phosphorylation.

We also studied the decrease in the activity of calcineurin in healthy T-cellstreated with 5 μM of Ca-ionophore and/or 80 nM phorbol ester (PMA). Our observation is that both PMA and Ca^{2+}-treatments contribute to the decrease of calcineurin activity of T-cell enriched PBMC without modulating the mRNA and protein levels of calcineurin. The calcineurin activity was more sensitive to Ca-ionophore than to the PMA treatment as the inhibition of the activity was higher for Ca-ionophore.

The use of cell-permeable PKC inhibitors suggests that cPKC α, β, and γ isoenzymes could be involved in the inhibition of the enzyme in human PBMC stimulated by PMA and Ca-ionophore. Gö6976, the selective inhibitor of cPKC isoenzymes, was able to reverse the inhibitory effect of PMA and Ca-ionophore applied alone or in combination on the calcineurin activity.

Our data also confirmed that Cabin 1 could be a transducer which mediates the PKC signalling towards calcineurin as the increasing phosphorylation state of Cabin 1 was observed in response to the stimulation by Ca-ionophore and PMA. The phosphorylation state of Cabin 1 was suppressed in the presence of GF109203X and Gö6976, inhibitors of cPKC and nPKC. This confirmed that Cabin 1 can play a role in the transduction of PKC signalling and in the inhibition of enzymatic activity of calcineurin in mononuclear cells.

CONCLUSIONS

✓ Calcineurin was found as a positive regulator of chondrogenesis in chondrifying chicken micromass cultures, as CsA reduced both the amount of cartilage and the expression of mRNAs of aggrecan and the chondrogenic transcription factor Sox9.

✓ Cartilage formation was inhibited by H_2O_2 in a concentration-dependent manner without loss of cellular viability or severe decrease of cell number. Expression of both the mRNA and the unphosphorylated protein Sox9 was decreased, while its phosphorylation was stimulated by either H_2O_2 or CsA.

✓ Oxidative stress decreased the activity of calcineurin but the phosphorylation of the member of MAPK family, Erk1/2 was extremely elevated either by 1 mM H_2O_2 or 2 μM CSA.

✓ The Erk1/2 inhibitor PD098059 attenuated the depletion of cartilage matrix, as well as decreased the expression and phosphorylation of Sox9 in the cultures treated with H_2O_2 or CsA.

✓ The chondrogenesis inhibiting effect of H_2O_2 is mediated, at least partly, by inhibition of calcineurin and by activation of Erk1/2.

✓ Both PMA and Ca^{2+}-treatments contribute to the decrease of calcineurin activity of T-cell enriched PBMC without modulating the mRNA and protein levels of calcineurin.

✓ PKC α, β, and γ isoenzymes mediate the enzymatic inhibition of calcineurin in mononuclear cells stimulated with PMA and Ca-ionophore.

✓ In this process hyperphosphorylated Cabin 1 is the transducer since Cabin 1 becomes hyperphosphorylated by the enzymatic action of certain PKC isoenzymes resulting in the inhibition of calcineurin in human PBMC.

Publication list

Publications the Ph.D. thesis is based on

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**These two authors contributed equally to the work.*

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Conferences (lectures)

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