Ph.D. THESIS

CALCIUM HOMEOSTASIS AND POLY(ADP-RIBOS)YLATION PATHWAYS IN CELLS OF CHICKEN HIGH DENSITY MESENCHYMAL CULTURES

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INTRODUCTION

Experimental models of in vitro *cartilage differentiation*

High density cell culture system (HDC) established from chondrogenic mesenchymal cells isolated from limb buds of 4-day-old chicken embryos is a well-known model of *in vitro* cartilage differentiation. This simple model provides information on the molecular steps leading to differentiation of chondroprogenitor cells to chondroblasts. Chondrogenic cells differentiate into chondroblasts when cultured in high density under appropriate circumstances. Chondroblasts start to secrete cartilage-specific matrix components, such as collagen type II and aggrecan and form significant amount of cartilage by the end of the 6-day-long culturing period. Detection of the expression level and the phosphorylation status of the cartilage-specific transcription factor Sox9, as well as monitoring the expression of the core protein of aggrecan are both reliable markers of chondrogenesis.

Steps and molecular regulation of in vitro *chondrogenesis*

In HDC, formation of cartilage starts with the recruitment of chondroprogenitor mesenchymal progenitor cells that after condensation and nodule formation differentiate into chondroblasts and chondrocytes. Differentiation takes place on day 3 of culturing. Nodule formation is partly regulated by transient appearance of Ca^{2+} dependent intercellular junctions like N-CAM and N-cadherin and gap junctions. The spindle-shaped mesenchymal cells turn into a round phenotype characteristic to chondrocytes on account of cytoskeletal changes. Cartilage-specific extracellular matrix can only be detected from culturing day 3 by metachromatic staining.

Sox9, the "master gene" of chondrogenesis encodes the transcription factor Sox9, responsible for the transcription of cartilage-specific matrix molecules, such as collagen type II, IX and X and the core protein of aggrecan. Expression and activity of Sox9 is regulated by intracellular factors. *Protein kinase A (PKA)*

has been described to phosphorylate Sox9, which gains a higher activity after this modification. Members of the calcium-sensitive *protein kinase C (PKC)* family have also been described as positive regulators of *in vitro* chondrogenesis, however, as yet no data are available concerning a possible direct interaction between PKC and Sox9.

The role of *phosphoprotein phosphatases*, allowing for the reversibility of protein phosphorylation is also fundamental. *Protein phosphatase 2A (PP2A)* is a negative regulator of chondrogenesis. Pharmacological inhibition of this phosphatase leads to an increase in the phosphorylation level of CREB, which in turn promotes chondrogenesis. The role of calcium-sensitive *protein phosphatase 2B (PP2B, calcineurin)* in chondrogenesis has only been described recently. Calcineurin dephosphorylates and activates NFAT, a factor that is known to facilitate chondrogenic differentiation. Pharmacological inhibition of calcineurin by *cyclosporine-A* leads to decreased cartilage matrix production.

Calcium-sensitive pathways regulate in vitro *chondrogenesis*

Changes in cytosolic Ca^{2+} concentration influence the activity of members of the PKC family (*i.e.* classic PKC) and calcineurin is also regulated by this mechanism. Among other cell types, Ca^{2+} homeostasis and the proteins involved (plasma membrane receptors, voltage and ligand gated Ca^{2+} channels, receptors of the endoplasmic reticulum, Ca^{2+} pumps etc.) have been described in pluripotent embryonic mesenchymal cells, but as yet no data are available concerning the Ca^{2+} homeostasis of chondroprogenitor mesenchymal cells.

Oxidative stress and hyaline cartilage

Cartilage is an avascular tissue, and chondrocytes are often exposed to *hypoxia*. For this reason, anaerobic pathways dominate their metabolism. Diseases affect-

ing articular cartilage (*i.e.* rheumatoid arthritis, osteoarthritis) lead to higher oxygen demand and at the same time a decrease of oxygen supply from synovial capillaries. On account of these processes an imbalance between oxidants and antioxidants takes place, hypoxia gets worse and a condition called *oxidative stress* affects chondrocytes. Such reactive oxygen species (ROS) can also normally be produced as by-products of the normal respiratory chain.

Poly(ADP-ribose)-polymerase and oxidative stress

When the antioxidant capacity of cells cannot eliminate the oxidants, the surplus ROS have detrimental effects on the plasma membrane and nucleic acids, cause oxidation and nitration of amino acid residues of proteins and degrade cartilage matrix components (proteoglycans and collagens). Besides these effects, derivatives of ROS (*i.e.* hydrogen peroxide and peroxinitrite) also cause DNA-strand breaks *(genotoxicity).* Beyond the direct detrimental effects accumulation of *poly(ADP-ribose)-polymers* synthesized by *poly(ADP-ribose)-polymerase (PARP)* has also been described. The enzyme, upon binding to the DNA-strand breaks, cleaves NAD^+ into nicotinamide and ADP-ribose, then polymerises the latter to form large branched poly(ADP-ribose)-polymers and attaches them to various nuclear proteins. This post-translational protein modification facilitates DNA-repair. Cells exposed to an extremely high extent of genotoxic effects (e.g. UV or γ irradiation) become necrotic. Under such circumstances PARP becomes overactivated with a resultant depletion of cellular NAD^+ and ATP, which eventually leads to cell death. A moderate DNA damage may lead to p53-activation and cells undergo apoptosis on account of the insufficient DNA-repair. These effects can be at least partially attenuated by *3-aminobenzamide* (3-AB), a pharmacological inhibitor of PARP.

PARP in arthritis

PARP can also get activated during local inflammatory processes (*e.g*. arthritis). Administration of the PARP-inhibitor 3-AB has lead to an attenuation of the symptoms in a mouse arthritis model. Since other PARP-inhibitors also had the same effect, a general role of PARP-mediated processes in arthritis has been implicated, although the exact mechanism has yet to be elucidated. Only sporadic data are available regarding a connection between PARP-mediated processes and *in vitro* chondrogenesis, the exact role of such pathways in the oxidative stress-induced responses in cells of HDC is unclear.

Oxidative stress and cytosolic free Ca^{2+} concentration in cells of HDC

ROS can exert their detrimental cellular effects by modulation of the cytosolic Ca^{2+} concentration, since elevation of basal cytoplasmic Ca^{2+} levels above a certain (cell type dependent) threshold disturbs the Ca^{2+} homeostasis, which in turn may lead to apoptotic or necrotic cell death. Furthermore, PARP has a direct effect on cytosolic Ca^{2+} concentration since it can regulate the activity of plasma membrane cation channels which, by elevating the cytoplasmic Ca^{2+} levels, could also contribute to apoptotic or necrotic cell death.

AIMS OF THE EXPERIMENTS

Various cell types exhibit characteristic changes in cytosolic Ca^{2+} concentration during differentiation. Although there are no data available concerning the relationship between the differentiation of chondrogenic mesenchymal cells into chondroblasts and changes in cytosolic Ca^{2+} concentration, on account of the large number of calcium-sensitive pathways involved, it might be reasonable to hypothesise that changes of cytosolic Ca^{2+} concentration characterise the differentiation process. Therefore, the aims of this study were to answer the following questions:

1. How does the cytosolic free Ca^{2+} concentration change during differentiation of cells of HDC?
2. What is the major source of cytosolic Ca^{2+}: the intracellular Ca^{2+} stores, or the extracellular space?
3. What are the effects of any kind of modulation of cytosolic free Ca^{2+} concentration on the differentiation process and/or the activity of calcineurin?

In the second part of the study we aimed to investigate another aspect of chondrocyte differentiation. As it was described earlier, chondrocytes have to maintain their function in hypoxia, and they frequently encounter reactive oxygen species. Therefore, we also wanted to answer the following questions:

4. How do reactive oxygen and nitrogen species affect *in vitro* chondrogenesis and cartilage differentiation?
5. Is there a relationship between oxidative stress-derived havoc and PARP-mediated processes in cells of HDC?
6. What can be the role of PARP under normal circumstances in cells of HDC?

MATERIALS AND METHODS

Cell culture

Distal parts of the limb buds of 4-day-old Ross hybrid chicken embryos (Hamburger–Hamilton stages 22–24) were removed and primary micromass cultures of chondrifying mesenchymal cells were established from a cell suspension with a density of 1.5×10^7 cells/mL. 15, 30 or 100 µL droplets of the suspension were inoculated on round coverglasses placed into plastic Petri dishes. Colonies were grown in Ham's F12 medium supplemented with 10% foetal calf serum.

Determination of cytosolic free Ca^{2+} concentration

Measurements were performed on different days of culturing using the calcium dependent fluorescent dye Fura-2. Fura-2-loaded cells were placed on the stage of an inverted fluorescent microscope and viewed using a 40× oil immersion objective. Measurements were carried out in Tyrode's salt solution in a perfusion chamber using a dual wavelength monochromator equipment. Fluorescence of Fura-2-loaded cells was measured using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Intracellular Ca^{2+} concentrations were calculated from the ratios of intensities ($R= F_{340}/F_{380}$) by using the following equation: $[Ca^{2+}]_i = K_D \cdot \beta \cdot \frac{R-R_{min}}{R_{max}-R}$

where K_D is the dissociation constant, R is the ratio of the fluorescence intensities, R_{min} is a ratio of intensities when Fura-2 does not bind to Ca^{2+} ions, R_{max} is a ratio of intensities when Fura-2 is completely saturated, β is a constant value. Test solutions were directly applied to the cells through a perfusion capillary tube using a local perfusion system.

Approaches to determine the Ca^{2+}-homeostasis of HDCs

The role of intracellular Ca-stores in the changes of the cytosolic Ca^{2+} concentration, the intracellular Ca^{2+}-pump inhibitor cyclopiazonic acid (CPA) was ad-

ministered. Caffeine, an agonist of ryanodine receptor (RyR) was administered at close proximity of cells during single cell measurements.

Spontaneous calcium transients were monitored using a laser scanning confocal microscope. Cells of high density micromass cell cultures were incubated for 1 h at 37 °C with 10 μM Fluo-4-AM. Calcium imaging was performed in normal Tyrode's solution. x–y analysis and line scan images were taken to monitor the fluorescence intensity during spontaneous activities.

In order to examine the effects of removal of Ca^{2+} from the extracellular fluid, cell cultures were fed a culture medium containing 0.8 mM EGTA for 12 hrs on day 2 or 3 of culturing. To assess the effect of elevated intracellular Ca^{2+}, cultures were fed with a culture medium containing calcium ionophore A23187 at concentrations of 0.1 and 5 mg/L for 1 h on culturing days 2 and 3. Activity of calcineurin was inhibited with the continuous application of 2 μM cyclosporine A started on day 1.

Determination of cartilage differentiation

Cartilage matrix was visualized by staining with dimethylmethylene blue (DMMB). The amount of sulphated matrix components was determined with a semi-quantitative method, by measuring the optical density of extracted toluidine blue bound to glycosaminoglycans in mature HDC.

Rate of cell proliferation was determined by monitoring the activity of the incorporation of ^{3}H-thymidine. Cellular metabolic activity was determined by MTT assay.

Rate of cell survival was determined by FACS analysis. Following treatments of either A23187 or EGTA, cultures were incubated with annexin-V DY647 reagent and/or propidium-iodide and rate of cell survival was determined using a flow cytometer. Annexin-V DY647 was monitored at 670 nm, propidium-iodide was measured at 620 nm. Analysis was performed with WinMDI 2.8 software.

RT-PCR analysis

Total RNA was isolated from cells of HDC and was converted into cDNA by using Omniscript reverse transcriptase kit and oligo(dT) primers. Amplifications of specific cDNA sequences were performed with specific primers that were designed using Primer Premier 5.0 software based on published chicken nucleotide sequences. PCR reactions were allowed to proceed in a final volume of 50 μL with primer annealing set at optimized temperatures in a programmable thermocycler. PCR products were analyzed by electrophoresis in 1.2% agarose gel containing ethidium bromide. Quantification of signal intensities was performed by using ImageJ software.

Preparation of cell extracts and Western blot analysis

For *total cell lysates*, cell cultures were harvested immediately after treatments on respective days of culturing and were suspended in homogenization buffer. Suspensions were sonicated to yield total cell lysate samples. For isolation of endoplasmic reticulum fraction of 3-day-old HDC, cells were homogenized using a Dounce homogeniser in a homogenization buffer containing HEPES and sucrose. After centrifugation at 150,000 *g* for 120 min, pellet containing endoplasmic reticulum vesicles (microsome fraction) was collected in lysis buffer. Samples were separated by 7.5% SDS–PAGE gel and proteins were transferred electrophoretically to nitrocellulose membrane. After blocking, membranes were incubated with the primary antibodies and then incubated with a secondary antibody. Signals were detected by enhanced chemiluminescence. Quantification of signal intensities was performed by using ImageJ software.

Enzyme activity assay of calcineurin

Activity of calcineurin was measured by the release of $^{32}P_i$ from ^{32}P-labelled protein phosphatase inhibitor-1. The assay mixture was incubated at 30 °C for 20 min. The reaction was terminated by the addition of 20% trichloroacetic acid

and bovine serum albumin. After centrifugation, $^{32}P_i$-content of the supernatant fraction was determined in a liquid scintillation counter.

Generation of oxidative stress and investigation of PARP-activity

For the generation of oxidative stress, 2-day-old HDCs were treated with variable concentrations of hydrogen-peroxide (0.1, 1 and 4 mM) or peroxynitrite (100, 300 and 600 μM) for 30 min. PARP-activity was inhibited by the administration of 5 mM 3-amino-benzamide (3-AB) prior to oxidative stress.

Immunocytochemical detection of poly(ADP-ribose)-polymers

Cells were fixed in ice-cold 10% acetic acid followed by an additional fixation in Saint-Marie solution. Aspecific binding sites were blocked in 5% horse serum and cell cultures were then incubated overnight at 4 °C with monoclonal anti-poly(ADP-ribose) antibody. Biotinylated horse anti-mouse IgG was used as secondary antibody and was visualised by streptavidin–Alexa-546. Nuclei were stained with DAPI.

In situ *detection of PARP activity*

The culture medium was replaced with PARP reaction buffer containing NAD^+. After a 1-hour-long incubation at 37 °C cultures were fixed. Aspecific binding sites were blocked and incorporated biotin was detected by streptavidin–Alexa-546. Nuclei were stained with DAPI.

Measurement of cellular NAD^+ content

Cells were extracted in $HClO_4$ and supernatants were used for measurements after the addition of the reaction medium. The absorbance of samples was de-

tected at 560 nm. Cellular levels were calculated from a standard curve generated with known concentrations of NAD^+.

Statistical analysis

Data were statistically analysed by Student's *t* test and changes were considered to be significant where $p < 0.05$.

RESULTS

Ca^{2+}-homeostasis in cells of HDC

Cytosolic free Ca^{2+} concentration follows a characteristic pattern during differentiation

Cytosolic free Ca^{2+} concentration was determined in Fura-2-loaded cells on different days of culturing. Basal level of intracellular Ca^{2+} concentration of chondroblasts was found to have an age-dependent pattern: initially, on day 0 Ca^{2+} level is low, then it slightly increases in parallel with the progression of differentiation. A 140 nM peak of the cytosolic free Ca^{2+} concentration was observed on day 3 of culturing. From day 4, Ca^{2+} level drops, however, it retains a slightly elevated concentration. Fluo-4-loaded 3-day-old chondroprogenitor cells also exhibit periodical increases in cytosolic free Ca^{2+}. Frequencies of these oscillations were similar in all the cells observed: the period was 4 ± 1.2 min^{-1}, maximum amplitudes were 15–20% higher than the mean basal fluorescence intensity.

The extracellular space proved to be the source of elevated cytosolic Ca^{2+} levels during differentiation of HDC

In order to reduce the concentration of free Ca^{2+} in the culture medium, EGTA was applied in equimolar (0.8 mM) concentration. EGTA treatment significantly decreased the cytosolic Ca^{2+} level to approximately 60% of that of untreated control cells, it caused a significant reduction of cartilage formation and a significant decrease in the mRNA and protein levels of Sox9 was observed. These data demonstrate that reduced intracellular Ca^{2+} level decreases cartilage formation, at least partly, via inhibition of cartilage differentiation.

Elevation of cytosolic Ca^{2+} concentration by Ca^{2+} ionophore has dual concentration-dependent effects on cartilage formation

A23187 Ca^{2+} ionophore was administered to cells of HDC at concentrations of 0.1 and 5 mg/L. Treatments with either concentration of the ionophore resulted in elevated cytosolic Ca^{2+} concentration, however, the two concentrations of ionophore applied had opposite effects on cartilage formation: following treatment with the lower concentration of A23187 an extensive cartilage formation occurred, while the higher concentration caused a marked inhibition of matrix production.

mRNA levels of Sox9 and aggrecan core protein showed a well-defined increase as a result of treatments with 0.1 mg/L A23187; on the contrary, we failed to detect any changes in the expression level of these factors in case of the higher concentration of the ionophore. On account of the apparent contradiction between these observations it might be hypothesized that the detected decrease in the matrix production caused by 5 mg/L A23187 may involve pathways other than that of Sox9.

Intracellular Ca^{2+} stores of cells in HDC contain a low amount of calcium

To investigate the role of intracellular Ca-stores CPA was administered to cells of HDC. Approximately 60 sec after the administration of CPA, cytosolic Ca^{2+} level started to increase very slowly. The slight elevation in cytosolic Ca^{2+} clearly showed that the intracellular Ca^{2+}-stores are not empty, however, the amount of stored Ca^{2+} is either low or the rate of leak is small. When administration of CPA ceased, a well-defined peak in cytosolic Ca^{2+} level could be observed owing to the entry of extracellular Ca^{2+} into the cytosol, reflecting on a possible activation of store-operated Ca^{2+} entry (SOCE) channels. CPA was also administered to the culture medium of HDC on days 2 or 3 for 12 hrs. This prolonged inhibition of the Ca-pump of smooth endoplasmic reticulum must have

resulted in a complete abolishment of intracellular stores, however, no detrimental effect on chondrogenesis could be observed.

The importance of Ca^{2+}-influx from extracellular space is further supported by investigating the endoplasmic reticulum ryanodine receptor (RyR) and the inositol-1,4,5-trisphosphate (IP_3) receptors. RyR was only detectable by Western blot analyses in samples containing separated endoplasmic reticulum fraction of HDC, thus though present, the low amount of RyR located in the endoplasmic reticulum probably does not significantly contribute to the elevation of basal cytosolic Ca^{2+}. On account of its low mRNA and protein levels a similar function can be concluded for IP3-R type 1.

Effects of EGTA, A23187 Ca^{2+} ionophore and CPA on rate of proliferation, mitochondrial activity and cellular viability of cells of HDC

Although the proliferation rate of cells in micromass cultures was significantly reduced and the rate of apoptotic/necrotic cells increased under the effect of 0.8 mM EGTA, the mitochondrial activity of cells was not affected. Cell proliferation was slightly stimulated by low concentration of the Ca^{2+} ionophore A23187 on day 3, whereas the high concentration slightly reduced the rate of cell proliferation on both days. However, none of the other parameters were affected under the effect of A23187 treatments. Administration of CPA did not influence the proliferation or necrotic/apoptotic rate of cells and it did not prove to be cytotoxic.

Expression and activity of calcineurin are regulated by cytosolic Ca^{2+} cencentration

When cytosolic Ca^{2+} level became lower as a consequence of reducing the extracellular Ca^{2+} concentration with EGTA, the activity of calcineurin decreased significantly on both days of treatments. Elevation of cytosolic Ca^{2+} level with A23187 had partly unexpected effects. On day 2 the elevation of Ca^{2+} concentra-

tion induced by the ionophore increased the activity of calcineurin, while on day 3, the ionophore-induced elevation of cytosolic free Ca^{2+} level resulted in a suppression of calcineurin activity. RT-PCR and Western blot analyses demonstrated that expression of calcineurin was only slightly modified by any kind of change of cytosolic Ca^{2+} concentration.

When the activity of calcineurin was inhibited by its pharmacological inhibitor CsA, the cytosolic Ca-peak observed in control HDC on day 3 of culturing was eliminated, although cells had a slightly higher basal Ca^{2+} level than those of the untreated controls. At the same time, matrix production was also decreased.

Oxidative stress and *in vitro* chondrogenesis

Oxidative stress-induced PARP activation contributes to suppression of matrix production

Hydrogen peroxide and peroxynitrite inhibited cartilage matrix production in a concentration dependent manner. Pretreatment with the PARP inhibitor 3-AB provided considerable protection against the observed inhibitory effects. Continuous application of 3-AB resulted in a substantial (over 50%) increase in cartilage matrix production when cells were not influenced by oxidative stress.

Hydrogen peroxide and peroxynitrite also inhibited the proliferation rate of cells of HDC; administration of 100 μM of peroxynitrite resulted in a 50% decrease as compared to untreated control cultures. Pretreatment of the cultures with 3-AB had no significant protective effect on proliferation. While treatments with peroxynitrite also resulted in a suppression of the metabolic activity of cells, PARP inhibition by 3-AB provided a significant protective effect against peroxynitrite.

Oxidative stress alters cytosolic Ca^{2+} levels in cells of HDC

As cytosolic Ca^{2+} concentration is a sensitive parameter influenced by extracellular stimuli, we investigated whether oxidative stress alters cytosolic free Ca^{2+} levels in chondrogenic mesenchymal cells. While low (0.1 mM) concentration of hydrogen peroxide resulted in a moderate elevation of the cytosolic Ca^{2+} level, administration of 4 mM hydrogen peroxide caused a significant increase in the intracellular Ca^{2+} concentration especially on day 3 of culturing.

Expression of cartilage specific markers is influenced by oxidative stress

mRNA expression levels of both Sox9 and core protein of aggrecan showed a significant decrease due to oxidative stress. Pretreatments with 3-AB provided considerable protection against the detrimental effects of hydrogen peroxide and peroxynitrite. Continuous application of 3-AB resulted in a profound elevation of the expression levels of both markers clearly reflecting the increased matrix production observed by metachromatic staining of HDCs without treatments of reactive oxidative species. Protein expression level of Sox9 was also decreased followed by treatments with hydrogen peroxide and peroxynitrite, which could also be attenuated by pretreatments with the PARP-inhibitor 3-AB.

Oxidative stress induced PARP activation in cells of HDC

PARP activity was detected by an in situ enzyme activity assay based on the incorporation of biotinylated NAD^+ into cells of HDC. In untreated control cells no PARP activity was detected, however, both peroxynitrite and hydrogen peroxide stimulated nuclear biotin incorporation which could be abolished by 3-AB pretreatments. Moreover, we also detected the presence of poly(ADP-ribose)-polymers, the product of the PARP-catalysed reaction: oxidative stress induced the nuclear accumulation of the polymer, whereas inhibition of PARP activity by 3-AB fully prevented polymer synthesis.

DISCUSSION

Ca^{2+} homeostasis in cells of HDC

We found that during the differentiation of chicken limb bud-derived chondrogenic cells to chondroblasts and chondrocytes, the cytosolic free Ca^{2+} concentration exhibits characteristic temporal changes: starting from lower (about 75 nM) Ca^{2+} levels, it reaches its maximum on day 3 of culturing with a peak of 140 nM and remains at a higher concentration (about 100 nM) until the end of the investigated 6-day-long culturing period. Basal Ca^{2+} concentrations of differentiating mesenchymal cells are comparable with that of epiphysis growth plate chondrocytes. Beside the long-term changes of basal cytosolic Ca^{2+} level, cells of HDC exhibit short-term, spontaneous periodical increases (oscillations) in cytosolic Ca^{2+} concentration, a phenomenon also characteristic to other differentiating cells. Intracellular Ca^{2+} oscillations may promote the activation of transcription factors (NFAT, CREB) needed for the differentiation of cells of HDC.

To determine whether the elevated cytosolic free Ca^{2+} level on day 3 of culturing is derived from extracellular sources or is released from intracellular stores, further experiments were performed. Free Ca^{2+} of the culturing medium was bound by the Ca^{2+} chelator EGTA. This treatment significantly lowered cytosolic Ca^{2+} levels on days 2 and 3, and cartilage formation was also significantly reduced by the end of the 6-day-long culturing period. Furthermore, a significant decrease in the mRNA levels of both aggrecan core protein and Sox9 was also observed underlying the decreased cartilage differentiation. Treatments with EGTA significantly reduced the proliferation rate without seriously affecting the mitochondrial activity in cells of HDC. Our data indicate that Ca^{2+} ions may play a role mostly in the onset and the early steps of differentiation, since removal of free Ca^{2+} from the extracellular space influenced chondrogenic differentiation until day 3 of culturing but not at later stages of culturing. These findings are further supported by studies published by other laboratories.

To investigate the effects of opposite changes of intracellular Ca^{2+} level, we have generated uncontrolled Ca-influx with the application of a Ca^{2+} ionophore. Low concentrations of A23187 raised intracellular Ca^{2+} levels in parallel

with increased cartilage differentiation and matrix production. Although high concentration of Ca^{2+} ionophore resulted in higher intracellular Ca^{2+} levels and led to a dramatic decrease in cartilage formation. Interestingly, the cartilage specific mRNA markers of Sox9 and core protein of aggrecan failed to show lowered expression, while the protein level of Sox9 was reduced implying the possibility of a translational regulation of this protein via Ca^{2+} sensitive pathways. Our results suggest that the elevation of intracellular Ca^{2+} levels above a precisely regulated physiological level may negatively influence *in vitro* chondrogenesis via Sox9-independent pathways.

Intracellular Ca^{2+} stores of chondrogenic cells were also investigated. Somewhat higher cytosolic Ca^{2+} levels could be observed following the treatment with CPA, probably owing to store-operated Ca^{2+} entry (SOCE) processes. In addition, IP_3-R type I and RyR were found weakly expressed by cells of HDC, and caffeine treatments (RyR-agonist) did not cause any significant change in the cytosolic Ca^{2+} level. Taken together, we conclude that intracellular Ca^{2+}-stores could play a less significant role in the modulation of the peak in cytosolic Ca^{2+} of HDC during their differentiation, but may take part in the regulation of basal cytosolic Ca^{2+} level.

Calcineurin can be either a subject or a regulator of changes in cytosolic Ca^{2+} concentration in cells of HDC

Activity of calcineurin responded to the manipulation of intracellular Ca^{2+} concentration in a cell-differentiation-stage dependent manner. On day 2, calcineurin responded to the changes of cytosolic Ca^{2+} concentration as it was expected, *i. e.* EGTA decreased, while the ionophore increased its activity. On the contrary, any kind of alteration of cytosolic Ca^{2+} level did result in a reduction its activity on day 3 of culturing. Since calcineurin has a positive role in the regulation of *in vitro* chondrogenesis of chicken micromass cultures, we suppose that the maintenance of its enzymatic activity requires a precisely set regulation of intracellular Ca^{2+} concentration. Under the effect of CsA, when the activity of calcineurin is lowered, differentiating cells failed to produce the peak-like increase in cytosolic free Ca^{2+} concentration on culturing day 3, although the basal Ca^{2+} levels were higher in CsA-treated cultures than in the untreated ones. This observation may imply an active regulatory role of calcineurin in the enhancement of Ca-influx responsible for the rapid transient elevation of cytosolic Ca^{2+} concentration of chondrogenic cells on day 3 via activation of plasmamembrane Ca^{2+} channels. This idea is supported by numerous data gained by different experimental models, in which calcineurin regulated the activity of various components of the Ca^{2+} toolkit.

Oxidative stress and PARP-dependent pathways in cells of HDC

Previous data suggest that many ROI and RNI species inhibit proteoglycan synthesis and induce cartilage matrix degradation. We demonstrated that peroxynitrite, similarly to the effects of hydrogen peroxide, also inhibited matrix production in cells of HDC in a concentration dependent manner. Proliferation rate, mitochondrial activity together with the mRNA- and protein expression of Sox9, were all decreased under the effect of treatments with hydrogen peroxide and peroxynitrite. Furthermore, an elevated basal cytosolic Ca^{2+} concentration in hy-

drogen peroxide-treated cells was observed reflecting on the possibility of the modulation of plasma-membrane Ca^{2+}-channel functions by ROI and RNI.

Both hydrogen peroxide and peroxynitrite seem to activate the 'DNA damage – PARP activation' pathway in cells of HDC. Presence of PAR polymers modulates the activity of enzymes involved in DNA repair. Excessive PARP activity depletes cellular NAD^+ and ATP stores eventually leading to cell dysfunction or cell death. On account of the inability of pretreatments with the PARP inhibitor 3-AB to prevent reduction of the cell proliferation rate, a PARP-independent pathway can be implicated for ROI and RNI in the modulation of the activity of signalling molecules regulating cellular proliferation. On the other hand, 3-AB pretreatments were able to prevent the decrease in mitochondrial activity in cells of HDC, probably implicating the role of PARP-dependent pathways in the regulation of cellular viability. NAD^+ depletion was only detected following treatments with high concentrations of peroxynitrite and hydogen peroxide. Activation of PARP was confirmed by the detection of PAR-polymers in oxidative stress-treated cells of HDC. We failed to detect polymers in untreated control cultures (*n.b.* a nucleolar signal was clearly visible), however, strong signals were recorded in cultures treated with both hydrogen peroxide and peroxynitrite.

Data in the literature are somewhat controversial concerning PARP-dependent pathways in chondrogenesis. Our results suggest that both hydrogen peroxide and peroxynitrite inhibit cartilage matrix production in a PARP-dependent manner, and probably trigger the 'DNA damage → PARP activation → cell dysfunction' pathway. Since cytosolic Ca^{2+} concentrations in oxidative stress-treated cells of HDC were comparably high as those followed by administration of the high concentration of the Ca^{2+} ionophore A23187 and both treatments resulted in a decrease of cartilage matrix production, we may conclude that oxidative stress effects on *in vitro* chondrogenesis, at least partially, via in-

fluencing Ca^{2+}-level modulators and/or Ca^{2+} dependent pathways in cells of HDC.

SUMMARY

Calcium homeostasis and poly-(ADP-ribose)-polimerase (PARP) dependent pathways represent two important aspects of *in vitro* chondrogenesis. The new results presented in this work are as follows:

- We have shown that the cytosolic free Ca^{2+} concentration of chondrogenic mesenchymal cells changes in a characteristic pattern parallel to *in vitro* cartilage differentiation with a definitive peak on day 3. This peak-like elevation of cytosolic Ca^{2+}, the major source of which proved to be the extracellular space, is indispensible to proper chodrogenesis.
- Chondrogenic cells are highly sensitive to any kind of modulation (especially the decrease) of the tightly regulated basal cytosolic Ca^{2+} concentration. Cellular proliferation proved to be the most Ca^{2+} sensitive parameter.
- Enzyme activity of the calcium-sensitive protein-phosphatase *calcineurin*, one of the positive regulators of *in vitro* chondrogenesis, is sensitive to the changes of cytosolic free Ca^{2+} concentration. Since calcineurin has been described to regulate a variety of cellular functions, it might act as a factor through which changes of cytosolic Ca^{2+} concentration take effect.
- We have shown that activation of PARP mediated pathways can be at least partially accounted for the decrease in cartilage matrix production as a result of oxidative stress.
- A basal PARP activity was detected in untreated control cells of HDC, which probably has a negative effect on cartilage matrix production.
- Oxidative stress could cause decrease of cartilage formation at least partially, via elevation of cytosolic Ca^{2+} concentration.

PUBLICATION LIST

Publications on which the doctoral thesis is based

Zákány R, Bakondi E, Juhász T, Matta C, Szíjgyártó Z, Erdélyi K, Szabó E, Módis L, Virág L, Gergely P.: Oxidative stress-induced poly(ADP-ribosyl)ation in chick limb bud-derived chondrocytes. *Int J Mol Med* 2007, 4:597–605. (IF: 1,847)

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Other publications

Zákány R, Szíjgyártó Zs, Matta C, Juhász T, Csortos C, Szűcs K, Czifra G, Bíró T, Módis L, Gergely P: 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* 2005, **305:**190-199. (IF: 4,148)

Juhász T, Matta C, Veress G, Nagy G, Szíjgyártó Z, Molnár Z, Fodor J, Zákány R, Gergely P: Inhibition of calcineurin by cyclosporine A exerts multiple effects on human melanoma cell lines HT168 and WM35. *Int J Onc* 2009, **34:**995-1003. (IF: 2,295[#])

Fodor J*, Matta C*, Juhász T, Oláh T, Gönczi M, Szíjgyártó Z, Gergely P, Csernoch L, Zákány R: Ionotropic Purinergic Receptor $P2X_4$ is Involved in the Regulation of Chondrogenesis in Chicken Micromass Cell Cultures. Accepted for publication: *Cell Calcium* (2009) (IF: 4,338[#]) **: these two authors contributed equally to this work*

Scientific values

Impact factor of publications on which the doctoral thesis is based: 6.185
Cumulative impact factor: 16.966
Number of independent citations: 7

[#]: impact factors refer to year 2007 (no new data available).

Conference attendances; oral presentations

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