Ph.D THESES

ROLE OF SER/THR SPECIFIC PROTEIN PHOSPHATASES IN THE REGULATION OF IN VITRO CHONDROGENESIS

Róza Zákány MD

UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
DEPARTMENT ANATOMY, HISTOLOGY AND EMBRYOLOGY
2001

1. INTRODUCTION

Diseases of articular cartilage and developmental malformations of appendicular skeleton are painful lesions seriously altering the quality of life. Deeper knowledge of the development and of the structure of articular cartilage, as well as discovery of the molecular mechanisms regulating the formation of cartilage templates of bony skeleton could promote the introduction of more efficient therapy of these illnesses.

Hyaline cartilage is mesenchymal in origin. Chondrogenic cells proliferate rapidly and condense into precartilage nodules at the beginning of chondrogenesis. Cartilage formation is influenced by cell-cell contacts via N-CAM and N-cadherin, different cytokines e.g. TGFb, FGF4, other paracrine mediators like PGE-2, or different hormones and hormone-like substances (retinol, parathormone, vitamin-D3). Beside these factors, composition of the extracellular matrix (ECM) and changes in the pattern of the expression of ECM-receptor adhesion molecules both play important roles in the modulation of the chondrogenic phenotype of cartilage precursor cells. Chondrogenic cells are elongated and are surrounded an ECM rich in hyaluronic acid, fibronectin and type I collagen. The characteristic adhesion molecule of these cells is the integrin type $\alpha5\beta1$ binding to fibronectin. Precartilage cells start to detach and become rounded soon probably due to the antiadhesive property of the transiently expressed tenascin. Parallel to this process there are changes in the ligand binding properties of the focal adhesion sites, which further influence the architecture of the cytoskeleton and enhance the rounding of chondrogenic cells. Cells begin to secret type II collagen and aggrecan, as well as link protein and cartilage matrix protein (CMP), which are characteristic macromolecules of the ECM of hyaline cartilage. This ECM provides a microenvironment indispensable for maintenance of the chondrocyte phenotype.

It is well known that intracellular level of cyclic adenosine monophosphate (cAMP) is elevating from the beginning of chondrogenesis. The primary mediator of the intracellular effects of cAMP is the cAMP dependent protein kinase (PKA), a member of the family of Ser/Thr specific protein kinases. Inactive form of PKA consists of two regulatory and two catalytic subunits. Catalytic subunits are released from the regulatory subunits and become activated if cAMP binds to the regulatory subunits. It is remarkable that the catalytic subunits are active only in case of phosphorylated status of Thr-197 amino acid residue. Different A-kinase anchoring proteins (AKAPs), which can bind the PKA holoenzyme to different subcellular compartments, play an important role in the modulation of the substrate specificity of PKA. One of the major substrates of PKA is the transcription factor cAMP response element binding protein (CREB). Phosphorylation of CREB on Ser-133 enhances its transcriptional activity. Another transcription factor playing fundamental role in the regulation of chondrogenesis, SOX9, is also phosphorylated by PKA. Phosphorylated form of SOX9 activates the promoter sequences of genes of type II collagen and aggrecan core protein more efficiently.

Reversibility of protein phosphorylation requires rapid and efficient dephosphorylation of phosphoproteins. It is depending on the activity of different cellular phosphoprotein phosphatases.

Classification of cellular PPs can be based on either their specificity for substrates or their sensitivity to inhibitors. PP1 dephosphorylates the β-subunit of mammalian phosphorylase kinase and can be inhibited by both inhibitors type I and type II proteins. PP1 has essential role in eukaryotic cells as the fact of the high evolutionary conservatism of the amino acid sequence of the catalytic subunit suggests it. Members of the PP2 family can dephosphorylate the α-subunit of mammalian phosphorylase kinase and can be inhibited neither by inhibitors type I nor by type II proteins. Three subclasses are distinguished in the PP2 family: PP2A can be inhibited by okadaic acid, PP2B (or calcineurin) is Ca- calmodulin dependent, while PP2C is Ca^{2+} or Mg^{2+} dependent.
PP2A forms heterotrimers in cells, the catalytic subunit (C) is associated with a regulatory A-subunit and variable B-subunits can bind to the dimers of A-C. PP2A plays crucial role in many different signal transduction pathways. Beside the regulation of the activity of variable transcription factors and other messenger proteins PP2A also influences the activity of a great number of protein kinases, among those PKA is one of the best known.

Several microorganisms produce inhibitors to PPs. Okadaic acid (OA) is a product of marine *Dinoflagellata* and was isolated from black sponges feed on such algae. OA is a polyether fatty acid, with good permeability on the cell membrane and inhibits the activity of PP2A efficiently in nanomolar concentrations. Activity of other type of enzymes such as protein kinases is not influenced directly by OA. Thus OA is an excellent tool for investigation of the role of PP2A in different cellular processes.

2. THE AIMS OF THE STUDY

A huge number of experimental data support the idea of the great importance of protein phosphorylation processes in the regulation of cartilage development. Indispensable role of variable protein kinases in the process of chondrogenesis is not a question under debate. Numerous substrates - among which there are several transcription factors - of such protein kinases already have been described, but there are no data available of the role of PPs during the formation of cartilage.
Thus our goals were the followings:

- Description of the changes of the activity of the major cellular PPs (PP1 and PP2A) during the onset of in vitro chondrogenesis in chicken micromass cell cultures
- Investigation of the possible role of those PPs in the regulation of cartilage formation with the aid of the application of variable phosphatase inhibitors to the cell cultures
- Identification of such substrate molecules which have role in the regulation of chondrogenesis and also are subjects of both phosphorylation and dephosphorylation processes simultaneously

3. EXPERIMENTAL MODELL AND METHODS

3.1. Chondrifying mesenchymal cell cultures

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 15 x 10^6 cell/ml. 100-100 µl droplets of the suspension were inoculated onto plastic coverslips placed into plastic Petri dishes. Colonies were nourished with Ham′s F12 medium, supplemented with 10% fetal calf serum and were kept at 37^{o}C in the presence of 5% CO_2 and 95% humidity in a CO_2 incubator. The medium was changed on every second day or after the OA-treatments.

3.2. Inhibition of activity of PPs and PKA

The role of PKA activity in the regulation of chondrogenesis was investigated with the aid of H89. Cells were maintained in the absence or the presence of 1, 5 or 20 µM H89 from the beginning of the first culturing day. In order to inhibit PP2A, 20 nM OA was added to the culturing medium for 4 hours on the second and third culturing days. After removal of the OA-containing medium, cell cultures were further fed with media without or with H89.

3.3. Light microscopic histochemistry, image analysis and ultrastructural studies

High-density cultures of different experimental groups were fixed in a 4:1 mixture of absolute ethanol and 40% formaldehyde at day 6 of culturing. Cultures were stained with dimethylmethylene blue (DMMB) dissolved in acetic acid, washed in acetic acid and were mounted in gum arabic. Some cultures were stained with aqueous DMMB. The cartilaginous areas were measured in 10 cultures of each experimental group in 4 independent experiments by computer-assisted image analysis. The size of the metachromatic cartilage matrix was recorded from 6.8x10^6 µm^2 rectangular areas of the central part of 25 different colonies of each experimental group. Data were statistically analysed with an ambiguity probe.

3.4. Biochemical analysis of collagen and proteoglycan content of micromass cultures

After 8 days of culturing, OA-treated and non-treated control colonies were mechanically removed from the coverslips, homogenised in liquid nitrogen and the soluble proteins were extracted. After extraction the samples were centrifuged, the supernatants were removed and extensively dialysed against distilled water and finally freeze-dried. To melt the collagens the remaining pellets were washed with 0.5 M acetic acid and digested with pepsin. The supernatants were further purified. Cartilage extracts were analysed on large-pore-size agarose-polyacrylamide composite slab gels. The gels were stained with toluidine blue in order to visualise the proteoglycans. The pepsin-digested samples were separated on 10% polyacrylamide minigels and proteins were transferred to a nitrocellulose membrane. The nitrocellulose membranes were blocked with 5% BSA and polyclonal antibodies raised against chicken-collagen types I and II, respectively were added for overnight. The membranes

were extensively washed and labelled with horseradish peroxidase conjugated anti-rabbit Ig and were visualised with 3,3'-diaminobensidine chromogen reaction.

3.5. Enzyme activity assays

Cartilage colonies were washed twice with physiological NaCl solution and sedimented by centrifugation (1200 g at 4°C for 5 min). Cell pellets were resuspended in homogenisation buffer containing protease inhibitory cocktail. For the assay of PKC activity the homogenisation buffer was supplemented with Triton X-100. After storing the samples at -70 °C, suspensions were sonicated. The homogenates were centrifuged at 10,000 g for 10 min and the supernatants were used for enzyme activity assays. PKA activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone IIA. PKC assays were performed as described. PP2A was assayed using ^{32}P-phosphorylase as phosphosubstrate. Assays were performed in 3 different samples of each experimental group in 4 independent experiments.

3.6. Immunoblots to variable protein kinases as well as to CREB and phospho-CREB

Samples for SDS-PAGE were prepared by adding 1/5 volume of fivefold concentrated electrophoresis sample buffer to cell lysates and boiled for 10 min. Samples were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. After transfer the membrane was blocked with 5% nonfat milk and 0.1% Tween 20 in phosphate buffered saline. After blocking, the membrane was washed and exposed to the primary antibodies. Primary antibodies applied were as follows: anti-PKA-Cα polyclonal antibody, anti-PKGIα/β polyclonal antibody and anti-PKCμ polyclonal antibody, anti-CREB and anti-P-CREB polyclonal antibodies in. Anti-P-CREB antibody recognizes the Ser-133 phosphorylated form of CREB. For detection of primary antibodies peroxidase coupled secondary antibodies were used with enhanced chemiluminescence reagent. Protein concentrations were determined with Bradford method using bovine serum albumin as standard.

3.7. Cell proliferation-assays

15 μl droplets of cell suspension were inoculated into wells of 96-well microtiter plates and cells were treated similarly to those of cultured in Petri dishes. 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 beta-counter. Measurements were carried out in 10 samples of each experimental group in 4 independent experiments. Data were statistically analysed with F-test.

4. RESULTS AND CONCLUSIONS

The aim of our studies was to elucidate the role of PP1 and PP2A in the regulation of *in vitro* chondrogenesis. Application of selective cell permeable inhibitors of PPs was a major approach in our experiments.

4.1. Effects of OA-treatments on chondrogenesis

The size of metachromatic cartilage matrix areas increased up to 215 % of controls by the 6th culturing day in micromass cultures, when OA was given to the culturing medium on days 2 and 3, as image analysis revealed.
There are data for tumour promoter effect of OA in different cellular systems, but we found normal electronmicroscopic morphology of chondrocytes and cartilage matrix, as well as collagen and proteoglycan compositions of the untreated controls and OA-treated cultures were alike. These results suggested that OA promoted the normal cartilage formation in micromass cultures.

4.2. Modulation of cell proliferation by OA

Although the time course of the changes of the proliferation activity in the untreated and OA-treated colonies was similar as it was assayed by ^{3}H-thymidine incorporation assays, but OA elevated the incorporation of the radioactive isotope on each investigated day. The highest increase was observed following the second OA-treatment, on day 3.

A very intense proliferation, followed by migration and condensation of chondroprogenitor cells is an essential and indispensable early step of chondrogenesis. Thus increase of cell proliferation resulting in higher number of condensing cells has enhancing effect on cartilage formation. Differentiation of cartilage cells chiefly happens simultaneously with the proliferation and condensation during first and second days of culturing in HD-cultures. Direct cell-cell contacts, endocrine and paracrine signals strengthen the commitment of precartilage cells, which first differentiate into chondroblasts by day 3 of culturing and start to produce cartilage-specific matrix components such as collagen type II and aggrecan. Cells become mature chondrocytes by the sixth culturing day that is the end of the culturing in our experimental system. It is also well known that transitional, short arrest of precartilage cells in mitosis facilitates the cartilage formation and it was demonstrated before that OA had this effect on other mitotic cell types. Taken together, two, virtually opposite effects of OA on cell proliferation both could promote chondrogenesis.

4.3. Changes of the structure of cytoskeleton under effect of OA

OA-treatments resulted in disorganization and submembranal condensation of actin filaments of chondroblasts. Numerous round cells appeared following OA-treatments.
It is well known, that disruption of actin cytoskeleton has a promoting effect on chondrogenesis. Since modulation of the structure of the cytoskeleton can trigger changes in the level of the transcriptional activity it could not be a surprise to suppose that changes in the shape of the chondrogenic cells may influence the activity of cartilage specific genes. Different enzymes, such as PKA for example, playing crucial role in variable signal transduction

processes, also can be anchored to cytoskeletal components, thus changes of cytoskeleton could modulate the activity of these enzymes.

4.4. Activity of PP1 and PP2A assayed in untreated or OA-treated cultures

Activity of PP2A decreased parallel to the onset of chondrogenesis, while activity of PP1 remained at a constant high level all over the culturing period. Application of 20 nM OA on days 2 and 3 resulted in a significant inhibition of the activity of PP2A, while activity of PP1 was almost unaffected. These data lead us to suppose an essential role of PP1 in the cellular activity of chondrogenic cells, because high doses (over 100 nM) of OA, inhibiting also PP1, were cytotoxic. Since application of 20 nM OA that inhibited only PP2A had different, chondrogenesis promoting effect, thus we suppose a regulatory role of PP2A in chondrogenesis.

4.5. Chondrogenesis when PKA is inhibited by H89

It is known, that one of the possible substrate molecules of PP2A is PKA and it is also demonstrated that phosphorylation of the catalytic subunit of PKA on Thr-197 is essential to the activity of the enzyme. Thus we investigated the possible relationship of PKA and PP2A in chondrogenic cells with the aid of the application of a cell permeable kinase inhibitor H89. Chondrogenesis was significantly inhibited (reduced to 4%), activity of PKA was decreased to 30% and cell proliferation was reduced to 10% when 20 μM H89 was given to the culturing medium of HDCs.

Since aggregation of chondrogenic cells was successfully carried out in the cultures treated with H89 but production of a cartilage specific matrix was not seen in the precartilage cell aggregates, we suppose that PKA plays a role in a later differentiation step, when precartilage cells become chondroblasts.

When OA was introduced to cultures pretreated with H89, cartilage formation was recovered until 14% of the untreated control and even activity of PKA was elevated compared to those cultures which were treated by H89 alone. Expression of the catalytic subunit of PKA did not change considerably in any experimental groups.

Our data provide evidence of the role of PP2A in regulation of the activity of PKA in chondrogenic cells. This hypothesis is also supported by data of others, when PP2A was demonstrated to inactivate the catalytic subunit of PKA via dephosphorylation at Thr-197.

4.6. Cell proliferation under effect of H89

Since inhibition of PKA with H89 significantly decreased the cell proliferation on each day investigated, we suppose a crucial role of PKA in the regulation of cell proliferation in chondrogenic cells. Decrease of cell proliferation was not modulated by simultaneous application of OA. Thus we concluded that the stimulatory effect of PP2A inhibition on proliferation of chondrogenic cells probably is based on the possible involvement of PP2A in PKA signalling pathway and PKA is an upstream mediator.

4.7. Changes of expression and phosphorylation of CREB when PP2A and/or PKA are inhibited

Unlikely to the data received of investigation of cell proliferation, formation of cartilage was significantly promoted by OA treatments even in case of those cell cultures which were pretreated with H89. Thus we suppose that PP2A may dephosphorylate such transcription factors which are substrates of PKA and are involved in the regulation of cartilage differentiation.

The major mediators of transcription modulatory cellular effects of PKA are the members of the family of CREB transcription factors. Activity of CREB is regulated by phosphorylation on Ser-133.

Immunoblots to CREB and phospho-CREB (P-CREB) revealed the followings: expression of CREB slightly decreased in H89-treated cultures and P-CREB was almost undetectable. OA-treatments had an opposite effect, amount of CREB slightly elevated and the level of P-CREB was considerably higher than in controls. These data suggest a role of PP2A in the regulation of PKA-CREB signal transduction pathway during *in vitro* chondrogenesis.

Others had described already that phosphorylation of CREB is a crucial event in both cAMP and BMP-2 induced chondrogenesis, as well in enchondral bone formation. PP2A and PP1 both can be involved in the dephosphorylation of CREB depending on cell type and experimental systems.

Since PP2A can dephosphorylate either PKA or CREB, thus inhibition of PP2A by OA can facilitate formation of cartilage in two ways: OA can prolong the phosphorylation status of the catalytic subunit of PKA which can phosphorylate CREB more efficiently and/or CREB could remain phosphorylated for a longer period.

5. SUMMARY

We have investigated the role of the two major cellular phospho-Ser/Thr-specific protein phosphatases PP1 and PP2A, as well as that of cAMP dependent protein kinase (PKA) in the regulation of *in vitro* chondrogenesis with the aid of the application of specific cell-permeable protein kinase and protein phosphatase inhibitors. Our results are the following:

1. We have modified the original high density cell culturing protocol according to the requirements of our experimental system.
2. We applied the phosphatase inhibitor okadaic acid (OA) at the first time for investigation of a normal differentiation process and reported stimulation of chondrogenesis.
3. With the aid of the morphological and biochemical analysis of the resulted cartilage we gave evidences of the enhancement of the normal cartilage differentiation instead promotion of any kind of tumour-genesis by application of OA.
4. The activity of PP1 was constant and high, while the activity of PP2A decreased with the progress of chondrogenesis.
5. OA, applied in nanomolar concentrations, had no effect on the activity of PP1. OA-treatment significantly decreased the activity of PP2A, which remained at this low level until the end of culturing. OA did not influence the PKA activities in the kinase assays.
6. Cell proliferation was stimulated by OA.
7. Inhibition of PKA activity with H89 resulted in almost complete blockage of cartilage formation. Application of H89 also caused significant decrease of the cell proliferation.
8. As OA-treatment increased the activity of PKA, we concluded that PP2A could dephosphorylate the catalytic subunit of PKA (PKA-C) during *in vitro* chondrogenesis.
9. Expression of PKA-Cα was influenced by neither OA-, nor H89-treatments.
10. OA, when applied simultaneously with H89 increased the low PKA activity, as well as enhanced the H89 inhibited chondrogenesis, but had no effect on lowered cell proliferation. Further PP2A-modulated mechanisms in the regulation of the cell proliferation of high density cell cultures could be involved.
11. Inhibition of PKA with H89 resulted in a decrease of the amount of phosphorylated CREB, while inhibition of PP2A caused an opposite effect. Therefore, we suggest that both PKA and PP2A play role in the reversible phosphorylation of CREB during *in vitro* chondrogenesis and CREB might be one of the transcription factors, which regulates cartilage formation.

6. PUBLICATIONS, LECTURES AND POSTERS

Papers in the subject of the thesis:

Zákány R, Bakó É, Felszeghy Sz, Holló K, Balázs M, Bárdos H, Gergely P and Módis L (2001): Okadaic acid-induced inhibition of protein phosphatase 2A enhances chondrogenesis in chicken limb bud micromass cell cultures. Anat. Embryol. 203: 23-34.
IF: 1,511

Módis L, Aydelotte MB, Hadházy C, Zákány R, Kocsis K and Hyttinen M (1993): Extracellular matrix organization studied by polarization microscopy in cartilage differentiating *in vivo* and *in vitro.* Orthop. Transact. J. Bone Joint Surg. 17: 839.
IF: 2,471

Zákány R, Szűcs K, Bakó É, Felszeghy Sz, Módis L and Gergely P (2001): Protein phosphatase 2A is involved in the regulation of protein kinase A activity and CREB phosphorylation during *in* v*itro* chondrogenesis.
The manuscript was submitted at the time of defending of theses and has been published in Exp. Cell. Res. 275, 1-8 (2002)
IF: 5,096

Other publications:

Kappelmayer J, Bacskó G, Birinyi L, Zákány R, Kelemen E and Ádány R (1995): Consecutive appearance of coagulation factor XIII subunit A in macrophages, megakaryocytes and liver cells in early human development. Blood 86: 2191-2197
IF: 8,782

Muratoglu S, Krysan K, Balázs M, Sheng H, Zákány R, Módis L, Kiss I and Deák F (2000): Primary structure of human matrilin-2, chromosome location of the *MATN2* gene and conservation of an AT-AC intron in matrilin genes. Cytogenet. Cell Genet. 90: 323-327.
IF: 1,604

Published abstracts:

Zákány R, Módis L and Ádány R (1994): Expression of tenascin during the early phase of human development. Cell. Biol. Internat.18: 553.
IF: 0.731

Lectures and posters in the subject of the thesis:

Módis L, Aydelotte MB, Hadházy Cs, Zákány R, Kocsis K and Hyttinen M: Extracellular matrix organization studied by polarization microscopy in cartilage differentiating *in vivo* and *in vitro*. Transactions of the 39. Annual Meeting, Orthopaedic Research Society, 1993, poster.

Zákány R, Módis L and Ádány R: Expression of tenascin during the early phase of human development. Fourth European Congress of Cell Biology, Prague, 1994, poster.

Módis L, Kocsis K, Zákány R and Dobai J: Submicroscopic heterogeneity of the extracellular matrix structure and its changes during the development. Fifth International Congress of Systematic and Evolutionary Biology, Budapest, 1996, lecture.

Zákány R, Bakó É, Felszeghy Sz, Gergely P and Módis L: The serine/threonine phosphatase inhibitor okadaic acid promotes cartilage differentiation *in vitro*. XVIth FECTS Meeting, Uppsala, 1998, poster.

Zákány R, Bakó É, Felszeghy Sz, Holló K, Balázs M, Gergely P and Módis L: Inhibition of protein phosphatase 2A enhances chondrogenesis via alteration of PKA-signaling pathway. XVIIth FECTS Meeting, Patras, 2000, poster and lecture.