THE LINK PROTEIN IN THE EXTRACELLULAR MATRIX OF CARTILAGE AND OTHER TISSUES

Ph.D. Dissertation

Mátyás Czipri, MD.

Department of Orthopedic Surgery
Clinical Centre
Faculty of Medicine, University of Pécs

Head of Doctoral School:	Gábor L. Kovács, MD, PhD, DSci
Program Director:	Péter Than, MD, PhD
Theme Leader:	Csaba Vermes, MD, PhD
Mentor:	Tibor T. Glant, MD, PhD, DSci

Pécs, Hungary

2015

ABBREVIATIONS USED

AGC: aggrecan
Agc1: mouse aggrecan gene
BMP: bone morphogenic protein
cDNA: complement deoxyribonucleic acid
CNS: central nervous system
COC: cummulus-oocyte complex
Crtl1: mouse cartilage link protein gene
Cspg: chondroitin sulphate proteoglycan
DNA: deoxyribonucleic acid
E: embryonic day
ECM: extracellular matrix
GAG: glycosaminoglycan
GAPDH: glyceraldehyde-phosphate-dehydrogenase
HA: hyaluronic acid, hyaluronan
LP: cartilage link protein
mRNA: messenger ribonucleic acid
PCR: polymerase chain reaction
PG: proteoglycan
RNA: ribonucleic acid
RT-PCR: reverse transcription-polymerase chain reaction
RT-QT-PCR: real time-quantitative-PCR
SED: spondyloepiphyseal dysplasia

1. INTRODUCTION

Chondrocytes like cells in other tissues, exist within an information-rich extracellular environment, consisting of extracellular matrix (ECM) molecules, a milieu which interacts with and modulates the activity of growth factors, hormones and ECM remodelling enzymes. Cell surface adhesion receptors connect structural information in the ECM to a complex cellular response mechanism in the cell interior. This complex cellular response mechanism will determine the chondrocyte gene expression, and hereby the assembly of the ECM.

The major macromolecular components of this ECM are the proteoglycan (PG) aggrecan (AGC), hyaluronic acid (HA), cartilage link protein (LP) and type II collagen. AGC forms some 35% (dry weight) of the proteins found in cartilage, although the the most abundant protein is collagen type II, which is present at up to 60 % by dry weight of the tissue. LP on the other hand represents only about 0.05 % of the net weight of cartilage. LP is a small globular glycoprotein in the ECM of hyalin cartilage, and its most characteristic function is its ability to bind HA. The molecule was first isolated from cartilage where it is abundantly present, and was termed as cartilage link protein.

Hundreds of AGC molecules associate with a single HA filament to form large PG aggregates with molecular masses of 10^8-10^9 Da. The LP, binding simultaneously to both HA and AGC, stabilizes the aggregate structure. The large PG aggregates are entrapped within the mesh-like network of type II collagen fibrils. The negatively charged glycosaminoglycan chains of AGC bind large amounts of water (about 70% of wet weight) and are responsible for the resiliency of cartilage, while the collagen fiber network is responsible for the tensile strength of cartilage. While the proper assembly of the ECM provides the cartilage with its mechanical characteristics, beyond the structural roles of ECM molecules they are also the participants and regulators of the environment in which chondrocytes are existing and differentiating. This way the ECM macromolecules are playing principal roles in chondrogenesis, chondrocyte differentiation and in the process of endochondral ossification. Mutation in genes encoding components of cartilage ECM may result in skeletal disorders, chondrodysplasias in mice and humans.

The human chondrodysplasias are a diverse and genetically heterogenous group of disorders of the skeletal development. Explosion of knowledge in the field of molecular genetic background of these disorders has led to a change in their classification by means of involving the affected genes and pathogenetic mechanisms to the classification. From our point of view the most important group is of the defects of extracellular structural proteins. This group includes some of the best characterized dysplasia families. Similarly to human chondrodysplasias, there are naturally occuring mutations in genes coding cartilage extracellular matrix structural proteins in animals. These mutations result in chondrodystrophic phenotype and often in death. In addition to these conditions there are several experimental animal models, mainly in mice, where targeted mutations in different ECM protein genes are created by means of different genetic approaches. These murine models are valuable since they allow studies of molecular mechanisms at a level of detail not possible in humans. Furthermore, the mice may serve as predictors of human diseases, as well as models in which specific questions concerning pathogenesis and novel therapies may be examined.

Although earlier the cartilage LP and other cartilage matrix protein genes were excluded as mutant loci in human chondrodysplasias, a study of mice with targeted mutation of the cartilage LP gene (*Crtl1*) showed a prominent function of LP in chondrocyte differentiation, cartilage ECM organization and skeletal development. The homozygous knockout mice ($Crtl1^{-/-}$) show dwarfism and flat face, whereas heterozygous mice ($Crtl1^{+/-}$) have no apparent phenotype. Most LP knockout homozygotes die shortly after birth due to respiratory failure, but those who survive develop progressive dwarfism and skeletal abnormalities characteristic of spondyloepiphyseal dysplasias. These mice have shortened long bones, flattened vertebrae, the antero-posterior axis of the skull is shortened with a dome-like skull and shortened snout.

2. AIMS

2.1 Genetic rescue of chondrodysplasia and perinatal lethal effect of cartilage link protein deficiency

Our aim was to investigate the function of LP in cartilage extracellular matrix and mouse skeletal development. To reach our goals we performed the following experimental studies:

- We generated LP transgenic mice with a cartilage specific expression vector. It was hypothesised that transgene-dictated LP overexpression will result in phenotypic changes.

- The transgenic approach necessitated the knowledge of the mouse LP coding sequence. The first step was to determine the mouse LP gene structure and cDNA sequence.

- In rescue experiments the transgenic mice were used to perform a genetic rescue by the introduction of the LP transgene into the genome of LP knockout mice. We hypothesised that the presence of the LP transgene in LP deficient mice will result in adequate protein production, and will lead to the rescue of perinatal lethality and to the „treatment" of chondrodystrophic phenotype.

- Different overexpressing transgenic lines were used in rescue experiments to determine potential influence of different levels of protein production on the phenotype. After the successful genetic rescue our goal was to investigate the survivors in details.

2.2 Link protein in extracartilaginous tissues

Previous studies reported the presence of cartilage LP in numerous non-cartilaginous tissues. Our aim was to define the tissue distribution of LP by systematically investigating the presence of LP in different tissues and organs of developing mice on the level of transcription and protein production as well. To reach our goals we performed the following experimental studies:

- Tissue distribution of LP transcripts was determined.

- Quantitative assessment of gene expression was carried out in different organs at different stages of mouse development.

- Tissue distribution of LP translational products was determined.

3. GENETIC RESCUE OF CHONDRODYSPLASIA AND PERINATAL LETHAL LETHAL EFFECT OF CARTILAGE LINK PROTEIN DEFICIENCY

3.1 Experimental procedures

3.1.1 Isolation and cloning of the murine LP gene

Cartilage from articular joints of neonatal mice were dissected, total RNA isolated and reverse transcribed (RT). Prior to reverse transcription, samples were digested with DNase I (Invitrogen, Carlsbad, CA) to eliminate residual genomic DNA from the RNA sample. First-strand cDNA was synthetized from 1 μg of total RNA primed by oligo-d(T) using SuperScript II reverse transcriptase (Invitrogen). The strategy for identification of the mouse LP gene was based upon RT-polymerase chain reaction (PCR), first using primers homologous in various species, and then using mouse specific primers. PCR products were directly cloned into the TA-vector (Invitrogen) and sequenced from both directions. Overlapping clones from the 5' and 3' ends were generated, using a 5' RACE system (Clontech, Palo Alto, CA) and 5’ and 3' Genome Walker (GW) systems (Clontech), and cloned into the pT-ADV vector. In order to identify the 5' transcription start site, primer extension was employed. A reverse primer in exon 1 (LP1) was end-labeled and used to both reverse transcribe mRNA from mouse chondrocytes and sequence a PCR product of genomic DNA corresponding to the LP promoter region. Both the [^{33}P]-ATP labeled RT product and the sequencing reaction were run in parallel on a 7% Long Ranger sequencing gel (FMC, Rockland, ME). For determination of intron-exon structure, the GW kit was employed using gene-specific nested primers located in the neighbouring exons. Resulting PCR products were sequenced and sequence alignments and analysis were performed using the Omiga^{TM} 2.0 computer package (Oxford Molecular Group, Hunt Valley, MD) or the NIH BLAST Server.

3.1.2 Generation of LP transgenic mice

In order to generate transgenic mice that over-express LP in cartilage, we designed a cartilage- specific transgenic expression vector. The vector was derived from pSPORT-1. The 5` box contains the type II collagen promoter. At the 3`-end, a 0.5 Kb fragment defined by the *Sma*I and *Xba*I sites consists of the SV40 promoter and poly-A adenylation site. The SV40 also serves a nonspecific enhancer function. At the very 3`-end of the vector, the 1.5 Kb fragment defined by the two *Xba*I sites consists of the type II collagen enhancer. Together these regulatory elements provide cartilage-specific expression. A 1758 bp-long cDNA fragment containing the entire LP coding sequence was blunt-end ligated into the polylinker site of the transgenic vector pSP/44-3, and the transgenic construct was used for microinjection (DNX Technologies, Princeton, NJ). Initially, ten founder mice were identified to harbour the transgene. From these mice, four independent transgenic lines were established and then two, a low (*Crtl1*^{TgA}) and a high (*Crtl1*^{TgC}), transgene and protein-expressing lines were used for further experiments.

3.1.3 Determination of genotype of transgenic and knockout mice.

Mice were genotyped by PCR using gene or neomycin (Neo)-specific primers with genomic DNA templates. Genomic DNA was isolated from tails by a standard method and a primer pair from different exons was used to identify the presence of the transgene. *Crtl1* heterozygosity was demonstrated by the presence of two PCR products (283 bp without and 1.88 Kbp with *Neo*) using gene-specific forward and reverse primers from intron 3 and exon 4, upstream and downstream from the knockout construct. In addition, homozygosity of the *Crtl1*-deficiency was confirmed by the presence of (i) two Neo-specific, and (ii) gene- and Neo-specific primer-generated PCR products, and (iii) the absence of the 283 bp PCR product.

3.1.4 Generation of polyclonal antibodies

Polyclonal antibodies were generated against murine cartilage LP by immunizing rabbits with synthetic LP peptides (Research Genetics, Huntsville, AL). Peptides were designed as LPro1 ^{142}VIEGLEDDTGV and LPro2 ^{341}KKHKLYGVYCFRAYN based upon their hydrophilic character and predicted antigenicity using methods incorporated in the Omiga 2.0 software package. Peptides were coupled to Keyhole Limpet hemocyanin, dissolved in PBS and emulsified in 1 ml complete Freund's adjuvant. Three rabbits were immunized intramuscularly with each peptide. Boosters were given every 2-3 weeks with peptide conjugates emulsified in incomplete Freund's adjuvant. Sera from immunied rabbits were collected weekly after the 5th injection. Antibodies from rabbit sera were purified on corresponding peptides bound to sulfolink columns. While all antibodies detected LP, polyclonal antibodies LPro1-R11 and LPro2-R18 were the most specific, showing essentially no background in Western blotting. These two antibodies were used for both Western blot analysis and immunohistochemistry in this study.

3.1.5 Tissue extraction, protein purification and Western blot analysis

For quantitative analysis, cartilage samples from 4-day-old newborn mice were pulverized under liquid nitrogen and then extracted with 4M guanidinium chloride in the presence of protease inhibitors as described. Extracts were dialyzed against water, freeze-dried and normalized for protein content. Crude extracts from skeletal tissue of wild type, transgenic, knockout and rescued newborn mice were rehydrated in Tris-acetate (0.1M, pH 7.2) buffer containing 0.15 M sodium chloride. Samples normalized to either protein or DMMB content were separated in 10% SDS-polyacrylamide slab gels under reducing conditions. Gels were electrophoretically transferred onto nitrocellulose membranes (BioRad, Hercules CA) and stained with rabbit antibodies to mouse LP (LPro1-R11 or LPro2-R18) followed by affinity purified and peroxidase-conjugated goat anti-rabbit antibody (Accurate Chemical, Westbury, NY). Enhanced chemiluminescence (Amersham) at a serial time range was used to detect immune reaction, which was then quantified using a PDI (Huntington Station, NY) gel scanner and an integrated software package program.

3.1.6 Quantitative assesment of gene expression

Crtl1 gene expression in cartilage of newborn mice with different genotypes, was quantified by real-time quantitative RT-PCR using the Smart Cycler System (Cepheid,

Sunnyvale, CA), and detection was carried out by measuring the binding of fluorescent SYBR Green-I to double stranded DNA. The PCR reactions were carried out in microtubes in 25 μl volume. The cDNA template (1 μl) of RT-PCR product was added to a PCR reaction mixture which contained final concentrations of 0.5 μM *Crtl1* specific forward and reverse primers, 1:50,000 dilution of SYBR Green-I stock solution (BioWhitaker Mol. Appl. Cambrex, Rockland, ME), 200 μM dNTP, 1.5 mM $MgCl_2$, and 2.5 units of Taq DNA polymerase (Promega, Madison, WI). For normalization, glyceraldehyde-phosphate-dehydrogenase (GAPDH) cDNA was amplified with specific primers. The fluorescence emitted by the reporter dye was detected online in real-time, and the threshold cycle (*Ct*) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The real-time PCR assay included two independent reverse transcribed RNA samples isolated from three newborn mice from three different litters.

3.1.7 Macroscopic and histological staining of skeletal tissues

Bones and cartilage of newborn mice were stained with alizarin red and alcian blue in 70% ethanol Specimens for histology were fixed with 10% buffered formalin, decalcified for 1-2 weeks and then embedded in paraffin. Tissue sections (6 μm) were trichrome-stained with safranin O, fast green and iron hematoxylin. For immunohistochemistry, mouse organs/tissues were embedded in OCT compound (Sakura Finetec, Torrance, CA), and 6-8 μm thick frozen sections on 3-aminopropyltriethoxi-silane-coated (Sigma) slides were fixed in ice-cold acetone for 5 minutes. Sections were washed with PBS, and pretreated with protease-free chondroitinase ABC (0.5 unit/ml; Seikagaku, Japan) at 37°C for 30 minutes followed by incubation with 10 % normal goat serum in PBS. Sections were immunostained with LPro1-R11 polyclonal antibody (1:100) diluted in PBS containing 1% normal goat serum for 1 hour. All immunostainings were carried out in room temperature. After washing, sections were treated with rhodamine-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in PBS for 1 hour. The slides were washed, mounted in Fluoromount (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and analyzed using a Nikon Microphot-FXA microscope or Eclipse TE 200 confocal microscope (Nikon, Garden City, NJ).

3.1.8 Breading protocol for genetic rescue

For rescue experiments, we generated $Crtl1^{-/-}$ animals that also carried the *Crtl1* transgene ($Crtl1^{-/-}Crtl1^{Tg+}$). Initially, homozygous transgenic females of lines A and C ($Crtl1^{TgA}$ and $Crtl1^{TgC}$; F9-F10 generations) were mated with heterozygous LP-null males ($Crtl1^{+/-}$), first to generate heterozygous $Crtl1^{+/-}Crtl1^{Tg+}$ offspring. Progeny (N1 generation) was genotyped by PCR, and $Crtl1^{+/-}$ mice carrying the transgene ($Crtl1^{TgA+}$ or $Crtl1^{TgC+}$) were selected for further breeding. Mice heterozygous for both endogenous and transgene *Crtl1* were intercrossed to generate $Crtl1^{-/-}Crtl1^{TgA+}$ or $Crtl1^{-/-}Crtl1^{TgC+}$ genotypes.

3.2 Results

3.2.1 Coding region and genomic structure of mouse Crtl1 gene

We determined the 2780 bp murine LP sequence, which has been deposited into GeneBank-EMBL (Accession number: AF 098460). The coding region contained a single open reading frame of 1,065 nucleotides encoding a protein of 355 amino acid residues. Comparative analysis demonstrated 99% homology with rat and 96% homology with human *CRTL1*. The 5' transcriptional start site is at -368 bp position relative to the translational start site. The mouse *Crtl1* gene is 68,099 bp-long on chromosome 13 between 90.138 and 90.206 Mb region immediately upstream of the versican gene (*Cspg2*) in a head-to-head orientation. Exon 1 of *Crtl1* was found to be non-coding, while exon 2 contains the translational start site, signal peptide and N-terminal sequence, exon 3 the A loop, exon 4 the B loop and exon 5 the B' loop and 3' untranslated sequences.

3.2.2 Link protein transgenic mice

Four cartilage LP transgenic founder mice showed stable transmission of the transgene to progeny, and were used to establish independent transgenic lines. Based on Southern blot and subsequent densitometric analysis, founder line C was determined to have the highest copy number, greater than 20 copies of the LP transgene. Founders A, B and D were determined to have less than 10 copies of the transgene. Because founder C had the highest copy number of the transgene, and Line A exhibited the best breeding properties, these two lines were maintained to establish homozygous LP transgenic lines, with different LP expression in cartilage. Cartilage-specific overexpression of LP has not resulted in phenotypic abnormalities. Normal tissue and organogenesis, normal endochondral ossification and skeletal development, normal birth ratio and growth were observed.

3.2.3 Genetic rescue from perinatal lethality

Two transgenic lines A and C with different protein production offered a possibly different outcome in genetic rescue. It was expected that those $Crtl1^{-/-}$ mice which were also carrying the transgene would survive. Indeed, while the $Crtl1^{-/-}$ (LP-deficient) mice without the LP transgene died soon after birth, most of the $Crtl1^{-/-}$ mice carrying the LP transgene survived and many reached adult age. The survival rates in N2 generations were different in the two rescue groups, and the rates directly correlated with the levels of the transgenic protein product. In the N2 generation, the survival ratio of $Crtl1^{-/-}Crtl1^{TgA+}$ mice was lower (4.2 %) than in the $Crtl1^{-/-}Crtl1^{TgC+}$ mice (14.4 %), both were lower than the expected ratio. The survival ratio was improved in the N3 generation, which almost reached the expected ratio in the $Crtl1^{-/-}$ mice rescued with the higher transgene-expressing line C.

3.2.4 Gene expression and protein production in wild type and genetically manipulated mice

Although heterozygous ($Crtl1^{+/-}$) mice exhibit normal skeletal growth and breeding characteristics both LP mRNA and protein expression was significantly less in these mice. The mRNA expression was less than half, while the protein expression was 54 ± 7% (n=10) when compared to the wild type expression. Neither mRNA nor LP was detectable in

homozygous newborn *Crtl1*-deficient mice, or in *Crtl1*$^{-/-}$ fetuses collected at E20.5. Although real-time RT-PCR could not confirm a higher expression of the transgenic transcript (mRNA) in the skeletal tissue of F6-F8 generations of mice from Line A than wild type mice, the translated product (protein) was approximately 50% higher (mean 48 ± 24 %; n = 11) in cartilage collected from 2-4-days old newborn than wild type mice. In contrast, LP mRNA level was at least 3-times higher in cartilage of transgenic Line C, which was accompanied by 153 ± 21% (n = 9) greater protein expression in newborn cartilage. In both rescue groups we detected decreased mRNA expression which was unexpectedly higher in the rescue group A, than in the rescue group C. In contrast the protein expression in rescue group A was only 14 ± 3% (n=10) of that in wild type mice, while in rescue group C it was found to be more than half of the wild type expression (56 ± 6%; n=10).

3.2.5 Phenotype of the link protein-rescued mice

There were evident and significant differences in a number of phenotypic characteristics when *Crtl1*-deficient mice rescued with the low or high transgene-expressing lines were compared. While the *Crtl1*$^{-/-}$*Crtl1*$^{TgA+}$ offspring survived, eventually, all *Crtl1*$^{TgA+}$-rescued mice developed more or less severe abnormalities in their skeletal structure. These newborn mice were smaller than their litter-mates, their snout was shortened, and the antero-posterior diameter of the head was shorter giving a dome-like appearance to the skull. The frontal and parietal bones and occipital squama were normally formed and mineralized, indicating that only cranial bones formed through cartilage templates are affected and bones derived from membranous ossification are normal. Their limbs were significantly shorter due to the 5-15% shorter length of the long bones at the time of birth.

Delayed appearance of the epiphyseal ossification centers of the long bones was noted. Beyond the changes described for the skull and long bones changes in the appearance of the axial skeleton were also present. The height of the vertebral bodies was significantly decreased leading to platyspondylia, which was accompanied by spinal deformities in the sagittal plane. A marked hyperlordotic curvature in the upper, and a hyperkyphotic curvature in the lower thoracic region of the spine created the massive spine deformities, which were consistently present in all mice rescued with the low amount of LP. The phenotypic anomalies found in rescue group A were similar to those described in *Crtl1*$^{-/-}$ mice, resembling massive chondrodystrophic abnormalities. The skeletal deformities remained apparent during skeletal maturation, and the dwarfism became even more evident in ageing mice. The growth plate structure of these mice was disorganized, although the columnar organization of hypertrophic chondrocytes was maintained. The hypertrophic zone of the growth plate was narrowed due to the reduced number of hypertrophic chondrocytes involved in the chondrocyte columns. A relative decrease in the number of chondrocytes was also present in the prehypertrophic and proliferating zone as well. The lower number of chondrocytes was associated with an extensive amount of the extracellular matrix, which was as rich in safranin O-stained PG as the growth plate cartilage in wild type animals. The bony trabecules appeared to be thinner in the calcification zone compared to wild type mice.

In contrast to the skeletal and growth abnormalities present in rescue group A, no skeletal deformities were seen in *Crtl1*$^{-/-}$ mice rescued by LP transgenic line C; except for mild

phenotypic abnormalities with the smaller epiphyseal ossification centers of long bones and platyspondylia, and reduced chondrocyte numbers in the growth plate accompanied by increased amount of ECM. Positive immunostaining of the cartilage in newborn mice proved the presence of the transgenic protein in the ECM of rescued mice.

3.3 Discussion

This chapter summarizes the results of a partial to complete rescue of cartilage LP deficiency-induced skeletal abnormalities and the elimination of perinatal mortality. The effect of the transgene, i.e. the rescue process, is dose-dependent and the results confirm that approximately 50% reduction of LP in cartilage does not cause significant pathology. In *Crtl1* null mice the cause of death after birth is directly attributed to the collapse of the upper respiratory tract lacking the functionally sufficient cartilage. In addition, in *Crtl1*$^{-/-}$ mice a spine deformity is present, with decreased antero-posterior diameter of the chest,which may negatively influence the cardiorespiratory functions in these mice. Even though in rescue group A, the 14±3% of the wild type levels of LP in cartilage appeared to rescue the respiratory problems allowing survival of *Crtl1* null mice in spite of their dwarfism and relatively severe skeletal abnormalities. This finding raises the question of how would this relatively low level of LP be able to restore the function of cartilage by only playing a role as a simple structural molecule?

The growth plate of *Crtl1*$^{-/-}$ mice was described to be highly disoganized in the original study, and the 93% of these mice died shortly after birth. The conclusion was drawn that the lack of LP primarily affects the differentiation from prehypertrophic to hypertrophic chondrocytes, which correlates well with the expression pattern of LP with the highest level in the prehypertrophic zone. The level of AGC was found to be significantly reduced in the *Crtl1*$^{-/-}$ cartilage confirming an important role of link protein in the deposition of PG aggregates. In contrast to the *Crtl1*$^{-/-}$ mice, the columnar oganization of chondrocytes was well recognizable in both rescue groups with a more significantly reduced number of chondrocytes in all zones of the growt plate in the rescue group A. In addition, the PG content of the cartilage ECM in rescued mice was not different in comparison with wild type mice. Any changes in the structure of the growth plate of rescued mice were mirroring the different levels of expressed protein.

As the decreased AGC level in *Crtl1*$^{-/-}$ mice can be attributed to the lack of a stabilizing LP function, one would expect that normal levels of LP are needed to reconstitute the aggregate structure, considering that LP and AGC are present in 1:1 stochiometry in the HA-AGC-LP tertiary complex. In contrast, in our rescue experiments, significantly lower amounts of LP were sufficient to restore the structure of cartilage. These findings may be related to the cartilage growth factor characteristics of LP. A peptide of 16 amino acids, cleaved from the N-terminal end of cartilage LP can function as a growth factor and is able to stimulate the expression of AGC, type II collagen and other small glycoproteins. This N-terminal peptide of LP may be involved in the homeostatic regulation and synthesis of matrix components during the normal turnover of cartilage ECM. A recent study detected increased expression of chondrocyte-specific transcription factor SOX9, prompted by the N-terminal peptide. Upstream in this complex regulatory signaling cascade, the link peptide was found to

bound to the bone morphogenic protein (BMP) type II receptor. This mechanism could, at least partially, explain the dose-dependent recovery of cartilage ECM in mice of rescue group A, where the relatively low LP production controlled by the transgene would provide a sufficient source for N-terminal peptide to be cleaved. Thus, the growth hormone effect of the N-terminal LP peptide would be restored, resulting in a more physiological composition of the cartilage ECM.

From the diverse and genetically heterogenous group of human chondrodysplasias, disorders having the most similarities with this mouse model belong to the spondyloepiphyseal dysplasia (SED) group. Within the SED group the spondyloepiphyseal displasia congenita seems to show the most analogy. This condition lies in the middle of the spectrum of severity, and characterized by flat face, cleft plate and short trunk short stature, although shortening of the limbs is present as well with delayed ossification. The spinal involvement includes kyphoscoliosis, dorsolumbar kyphosis and increased lumbar lordosis with platyspondylia and anterior wedging of vertebrae. It is difficult though, to find similarities on the molecular level between the human SEDs and cartilage LP deficiency in mice. To date, there is no human skeletal disorder mapped to the LP gene. However, it can`t be roled out that a certain class of chondrodysplasia may be due to the defect of the link protein gene. Based on our animal model, a spondyloepiphyseal phenotype could be predicted with autosomal recessive inheritance.It is also possible, that the disfunction of cartilage LP in human has a catastrophic effect and might terminate life early in utero, making the recognition of an osteochondral disorder impossible. Our investigations to explore the presence and possible roles of LP in noncartilaginous tissues will be discussed in the next chapter.

Our results suggest that LP may have at least two functional roles in cartilage. One function is to provide structural stability of the cartilage aggregate by interacting with AGC and HA. Formation of stable cartilage aggregates is essential for the function of the growth plate, but approximately 50% LP expression level in a normal growth plate is sufficient for this stability. The 50% expression level is equivalent to that we could detect in heterozygous LP-null mice where normal phenotype is observed. It is noticeable though, that mice in rescue group C have as much LP protein expressed as the heterozygous knock out mice and still can have some mild chondrodystrophic changes which are never present in *Crtl1*$^{+/-}$ mice. The mRNA expression was also well comparable in the above mentioned two groups. These findings suggest that not only the absolute amount of LP mRNA or protein is important in the regulation and assembly of cartilage ECM, but the gene locus as well with its possible regulatory mechanisms. An unexpected additional function is to promote cartilage development by increasing levels of PGs and perhaps other cartilage matrix proteins such as type II collagen. The restoration of this function, however, might require less amount of LP, as the 14±3 % of the wild type expression level is able to rescue homozygous LP knockout mice from perinatal death and is able to partially restore cartilage assembly.

4. LINK PROTEIN IN EXTRACARTILAGINOUS TISSUES

4.1 Experimental procedures

4.1.1 Identification of transcriptional products

To determine tissue distribution of *Crtl1* transcripts, total RNA was isolated from various mouse organs and tissues at different ages, and reverse transcribed as described for cartilage. Total RNA was isolated from 10.5 days old whole embryos; from skeletal tissue, brain, liver and heart of 16.5 days old embryos; and from skeletal tissue, brain, liver, lungs, heart, kidney, spleen and intestines of 20.5 days old embryos. The same organs were used for RNA isolation in newborn and adult mice, with the additional four organs of testis, eye, ovarium and uterus in adult age. To identify reverse transcribed LP mRNA in various organs, RT-PCR was employed using LP specific primer pairs different exons. PCR products were separated in a 1.5% agarose gel and visualized by ethidium bromide staining. Representative PCR products were isolated from gels (Quiaex II gel extraction kit, Qiagen Inc., Valencia, CA) and sequenced using an ABI 310 genetic analyzer (Perkin Elmer, Branburg, NJ). For Northern dot-blot analysis, total RNA was isolated from 20.5 days old embryos (E20.5) and mRNA purified using Oligotex mRNA kit (Qiagen Inc). Two μg of this oligo-d(T)-purified mRNA in 2 μl final volume was dotted on GeneScreen Plus membrane (New England Nuclear, Boston, MA), baked at 80^{o}C in a vacuum oven, prehybridized, and then hybridized with a ^{32}P-dCTP-labeled 775 bp long PCR product generated by LP specific primers.

4.1.2 Quantitative assessment of gene expression

Crtl1 gene expression in different wild type mouse organs at different ages was quantified by real-time quantitative PCR using the Smart Cycler System (Cepheid, Sunnyvale, CA). Total mouse embryo at 10.5 days; skeletal tissue, brain, liver, heart at E 16.5; skeletal tissue, brain, liver, lungs, heart, kidney, spleen and intestines at newborn and adult age were used. The detection was carried out by measuring the binding of fluorescent SYBR Green-I to double stranded DNA. The PCR reaction mixtures were prepared as described previously. The real-time PCR assay included two independent reverse transcribed RNA samples isolated from three embryos, or organs and tissues of three animals.

4.1.3 Tissue extraction, protein purification and Western blot analysis

Crude extracts from various organs of 40 adult mice were prepared in 4M guanidinium chloride containing protease inhibitors as described for cartilaginous tissues previously, except that tissue samples were homogenized. As the *Crtl1* transcript was detected in many organs, but the translation product was detected only in crude extracts from cartilaginous tissues, subsequent purification was performed. This procedure resulted in 2-8 mg lyophilized material from the 600 mg tissue extracts, which was used to detect LP in different organs. These purified samples were rehydrated in Tris-acetate buffer containing 0.15 M sodium chloride and the immunodetection of LP was carried out by Western blot analysis as described previousy for cartilage. Immunohistochemistry was carried out on frozen sections and was described in details before.

4.2 Results

4.2.1 Tissue distribution and quantitative analysis of link protein transcripts

Total RNA was isolated from E10.5 whole embryos, various organs of E16.5 and E20.5 embryos, newborn and adult mice. RT-PCR analysis demonstrated the presence of *Crtl1* transcript in every organ tested in all age group. At the earliest time point, which was set to be prior to the beginning of cartilage formation at E13.5, LP transcripts were well detectable in the E10.5 whole embryos. At E16.5 the size of the mouse embryos allowed us to collect different organs separately. Those organs, which represented reasonable source for tissue samples (skeletal tissue, brain, liver, heart) all had well detectable amounts of *Crtl1* transcripts. At later sages of mouse development (E20), and after birth (newborn, adult) all investigated organs showed *Crtl1* expression. Although RT-PCR does not allow us to draw quantitative conclusions, the mRNA expression in skeletal tissue and brain samples always seemed to be well detectable. Liver samples on the other hand usually gave weak bands on gels, which might be due to the higher risk of enzymatic degradation in these samples. Interestingly, *Crtl1* transcripts were wery well detectable in adult reproductive organs and in the eye. Similarly to RT-PCR results, Northern dot-blot hybridization detected the *Crtl1* transcript in all tissues of E20 embryos.

For quantitative detection of *Crtl1* transcript levels the mRNA samples of different organs were reverse transcribed and amplified by RT-QT-PCR (n= 9-11 samples for each organ). The spatial and temporal differences in the *Crtl1* gene expression were expressed relative to the GAPDH expression. Relatively high mRNA expression was found in the E10.5 mouse embryo, prior to the commencement of cartilage formation. The highest expression was present in the cartilaginous skeletal tissue of the E16.5 mouse embryos at the period of the fetal growth and development which was nearly seven-times higher than at newborn age, and 89-times higher than in adult cartilage. Similar expression pattern was found in heart, as the *Crtl1* expression decreased by 30% between E16.5 and newborn age, and dropped significantly by 33 times between newborn and adult age. In contrast the levels of *Crtl1* mRNA changed in the opposite direction in mouse brain, with equally low expression at E16.5 and newborn age increasing more than 5 times by adult age, which was eventually found to be higher than the expression in cartilage at that age. *Crtl1* mRNA was also highly expressed in newborn lung, reaching more than two third (69%) of the expression in cartilage. This high expression in the respiratory tract at newborn age points to the importance of LP in the cartilage-containing upper respiratory tract and in the respiratory adaptation at that age. In adult mice the *Crtl1* expression in the lungs remained relative high and represented 35% of the expression in cartilage. Intermediate levels of expression were seen in intestines and kidney (18.5% and 4.5% of skeletal tissue, respectively) at newborn age, which was higher than the expression of these organs in adult age and may be attributed to the increasing function of these organs after birth. Although the *Crtl1* expression in the liver was well detectable at E16.5 (0.3% of skeletal tissue), the expression level remained very low in newborn and adult age. Interestingly, spleen was found to be expressing *Crtl1* on a relatively high level in adult mice (21% of skeletal tissue), which was 3 times higher than in newborn, and exceeded the gene expression of the adult kidney and intestines.

4.2.2 Tissue distribution of link protein translational products

The presence of LP protein was also confirmed by Western blot analysis in protein extracts from different tissues and organs of adult mice. The abundant presence of LP in cartilaginous tissues allowed the immunodetection of the protein even in crude extracts by Western blot, while other tissues needed to be further purified for adequate detection. Accordingly, presence of LP protein was confirmed in purified protein extracts from all the tested tissues and organs of adult mice.

To confirm the spatial tissue distribution of the translated *Crtl1* product, frozen sections from different organs were prepared and stained with rabbit polyclonal antibody LPro1-R11. Although at different levels, the LP was detectable in every organ tested by immunohistochemistry. At the earliest time point of immunohistochemical detection of LP in an E13.5 mouse embryo it was shown to be present in the primordial cartilaginous vertebrae and baso-occipital bone. Extracartilaginous detection of LP at the same age included the dermis of the skin, and the aorta. The immunopositivity of the E19.5 fetal kidney was also well demonstrated.

4.3 Discussion

Although LP is abundant in cartilaginous ECM, where it simultaneously interacts with AGC and HA, the *Crtl1* gene encoded LP was found in a number of tissues other than cartilage. In this study, which was the first systematic search for LP transcripts and translational products in a mammalian system, we systematically tested many organs for the expression of *Crtl1* mRNA and protein during embryonic development and in ageing animals. The presence of *Crtl1* mRNA expression in mouse embryos before the cartilage formation commences, as well as in a large number of non-cartilaginous tissues clearly demonstrates its ubiquitous presence, that has been known from several previous observations. In our systematic search LP transcripts and protein were detected in skeletal tissue, brain, liver, lungs, heart, kidney, spleen, intestines, testis, eye, ovarium and in uterus in four ages including adult and newborn mice and mouse embryos. Our results regarding the quantitative detection of *Crtl1* gene expression revealed different temporal expression patterns, which could indicate increasing or decreasing importance for LP at different time points in different tissues. Higher gene expression can indicate a more important role either in developmental processes or in the function of an organ or tissue. For example, the increasing expression pattern of *Crtl1* in mouse brain suggests higher importance for LP in the adult CNS, than in the developing brain. Cartilage LP is known to be present in brain and in spinal cord grey matter. It is exclusively produced by neurons and serves as an important building block in the assembly of perineuronal net, a condensation of ECM around neurons and dendrites. In contrast, the declining expression pattern of *Crtl1* in heart between the intrauterine life and adult age suggests more involvement in early heart development for LP. This is in line with the reported *Crtl1* expression by endocardial and endocardium-derived cells in the developing murine heart. Another functional role was addressed to LP in the mouse follicular development. LP was shown to be produced by cumulus cells, oocytes and granulosa cells and may facilitate the expansion of the cumulus-oocyte complex (COC) by stabilisation of HA rich ECM. Indeed, in our series *Crtl1* protein and mRNA was well

detectable in mouse reproductive organs including ovarium, uterus and testis as well.*Crtl1* was found to be an excellent candidate neurulation gene in chicken embryos. The LP gene can be a regulator of cell behaviour which results in the formation, elevation and convergence of the epidermal ectoderm during neurulation. Although a large number of embryos was investigated we could not find any consistent extra-skeletal disorder, but in some of the *Crtl1*$^{-/-}$ embryos we have found signs of a possible brain developmental failure resembling incomplete neurulation process.

In finding possible functions of LP in noncartilaginous tissues it is without doubt, that molecular partners have to be searched for. Based on the HA binding characteristics and the well known function of LP in cartilage, the best group of candidate molecules possibly interacting with LP in a ternary complex structure are the chondroitin sulphate PGs (LP-Cspg-HA). AGC, previously thought to be exclusively present in cartilage where it`s HA binding is facilitated by LP, has been found in several noncartilaginous tissues. AGC has been shown to be present in the extracellular matrix of developing and adult brain, where it is mainly synthesized by neurons. AGC, similarly to cartilage LP, is present in the perineuronal nets and is upregulated at the time of perineuronal net formation. The molecule is binding to HA, stabilized by LP and cross-linked by tenascin-R is one of the main components of the ECM in the CNS and is almost the exclusive PG in the perineuronal nets. The first demonstration of AGC in embryonic chick heart revealed unique expression pattern including all the major sites, where mesencymal cells contribute to the future separation of the primary heart tube. AGC is also present in other noncartilaginous tissues, like tendon, microvascular pericytes, and in human sclera.Versican, the other large HA binding chondroitin sulphate PG known to be abundantly present in the ECM of a variety of tissues was originally isolated from human fibroblasts. There are several tissues to date, where versican was found to be present. Systematic search for the versican gene (*Cspg2)* expression in mice revealed high expression levels in embryonic development with a declining tendency from day E13. It was detected in adult mouse tissues as well: brain, lung, spleen, heart, skeletal muscle, skin, testis, kidney. In the CNS versican is synthetized by astrocytes and oligodendrocytes. These observations support the theory that cartilage LP may interact with these large PGs in a number of tissues in different time points.Neurocan, another member of the large aggregating Cspg family is mainly present in the CNS. Experimental evidence seems to support that HA, neurocan and cartilage LP aggregates are likely to form in developing brain.

The startpoint of our investigations was a well characterized interaction with distinct function of the HA binding molecules (AGC and link LP), both of which was originally thought to be cartilage specific. The result of this work is in concordance with other observers and offers a new and extended way of thinking about these ECM molecules. HA binding PGs as well as LP are represented in many tissues and in many ECMs. These molecules may have pivotal roles in developing embryonic tissues, in the CNS and in many other areas. Similarly to the AGC-LP interaction in cartilage, some special molecular interactions between these molecules is already characterized, but a number of new roles and interactions need to be elucidated offering new opportunities for research.

5. NOVEL FINDINGS

5.1 Genetic rescue of chondrodysplasia and perinatal lethal effect of cartilage link protein deficiency

- We determined the coding region and genomic structure of mouse cartilage LP gene. The *Crtl1* resides on chromosome 13, and the coding region contains a single open reading frame of 1065 nucleotides encoding a protein of 355 amino acid residues. *Crtl1* shows 96% homology with the human CRTL1 gene.

- LP transgenic mice were created with a cartilage specific expression vector, and two LP transgenic mouse lines were established with stabilized LP overexpression. The transgenic lines served as the base for the animal model of genetic rescue, in which the role of LP was addressed. The protein production of LP transgenic mice was quantitatively measured and expressed relative to the wild type protein production. It was shown that the two transgenic lines differed in the LP protein expression. The protein production was approximately 50% and 150% higher relative to the wild type production in the transgenic line A and C respectively.

- The LP transgenic mice (line A and C) were successfully used in a genetic rescue experiment by substituting the missing LP in knockout mice. The lethal effect of the lack of LP was rescued by the LP transgene, the LP knockout homozygous mice carrying the transgene survived and reached adult age. Beyond the survival of these mice, the chondrodystrophic changes and dwarfism was reduced or eliminated by the LP transgene, modelling a successful gene therapy.

- Survival rates and phenotypic appearance were shown to be different in rescued mice depending on the transgene used for the genetic rescue. Mice rescued by the transgenic line A showed lower survival rates, while as in rescue group C the survival rates almost reached the expected Mendelian ratio.

- Animals in rescue group A were smaller, with shorter limbs, the antero-posterior diameter of their head was shorter, they exhibited platyspondylia and had massive thoracic spine deformities in the sagittal plane. In contrast, the chondrodystrophic phenotype was not present in rescue group C.

- The measurement of LP protein expression in rescued mice revealed, that the phenotypic appearance and changes in osteochondrogenesis were dependent on the amount of the expressed cartilage LP. 14% of the wild type LP expression was sufficient to rescue LP deficient mice from perinatal death, although these mice exhibited severe chondrodysplasia. On the other hand, mice with 56% of the wild type level of cartilage LP not only survived but grew normally without skeletal deformities.

5.2 Link protein in extracartilaginous tissues

- Systematic search for cartilage LP transcripts and protein in various noncartilaginous tissues in different time points of mouse development revealed its ubiquitous presence in tissues other than cartilage. This was the first systematic search for cartilage LP in a mammalian system.

- Cartilage LP is expressed during embryogenesis, even before cartilage formation commences.

- Quantitative analysis of cartilage LP gene expression revealed potentially different importance of LP in different organs in different time points. Some expression patterns suggest increasing importance with upregulation at initiation of the function of an organ.

- LP expression remains dominating in cartilage containing organs at all ages of mouse development with significant increase in the the lungs at birth.

- LP expression in brain remains limited during mouse development, but reaches high levels at adult age and is overcoming the expression even in the cartilaginous tissues.

- LP expression in the developing heart is the second highest in utero after cartilage during organogenesis, and the declining expression after birth suggests less importance at adult age.

- Protein products of the mouse cartilage LP were identified in tissue extracts and in tissue sections with polyclonal antibodies. The LP was found to be present in skeletal tissue, brain, liver, lungs, heart, kidney, spleen, intestines, testis, eye, ovarium, uterus, aorta and skin.

6. ACKNOWLEDGEMENTS

My highest appreciation belongs to Professor Tibor T. Glant for his continuous lead, strong support and mentorship. It would have been impossible to accomplish this work without his guidance and help. He and his entire team at the lab at the Department of Orthopedics, Section of Biochemistry and Molecular Biology at Rush University (Chicago, IL) provided me with the professional atmosphere, that was essential to conduct my research. I would like to thank to Professor Katalin Mikecz, Csaba Vermes, Sonja Velins, Jeffrey Otto, Reinout Stoop, Rajesh V. Kamath, István Gál, Andrew Nesterovitch, Yanal Murad, Kevin Kolman and Jian Zhang for giving me a hand to finish my work. Special thanks to my good friend Tamás Bárdos for his continuous help, advice and support, which made those years spent in Chicago so valuable.

I am thankful to all of my former colleague from my mother institution, the Department of Orthopedic Surgery at the University of Pécs. I would like to express my gratitude to Professor Árpád Bellyei, my progressive-thinking chief at the beginning of my carrier, for giving me the unique opportunity to conduct scientific research in a professional environment, and for his continuous encouragement towads academic achievements.

I am grateful for my program director, Professor Péter Than and my theme leader, Csaba Vermes for their professional support and guidance. Their help was invaluable to me at the finishing stages of my work.

Finally, I express my most sincere thank to my family, my wife, my mother and my brother for their love, sacrifice and unconditional support.

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