Molecular biological approach of osteoporosis and surgical bone substitution

Doctoral thesis

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INTRODUCTION

Osteoporosis (OP) is a common disease characterized by the deterioration of bone quality and quantity yielding increased risk of low trauma fractures due to the decreased molecular and biomechanical proprieties of the affected bone. Previous studies showed that the heritability of the bone quality and quantity parameters is around 60-80% but the exact role of the participant genes int he pathomechanism is still unclear. More than 200 candidate genes influencing the bone biology and the disease have already been reported and many gene variants have been previously described as significant predictors of fracture risk and/or bone mineral density. Allelic variants, especially single nucleotid polymorphisms (SNPs), have got crucial role in the genetic background of OP. Positive findings of some candidant genes and SNPs have been confirmed by further studies while regarding other genetic associations conflicting data can be found in literature. Previously, less investigated genes came to our attention in our previous studies about the relationship between deer antler development and human OP. Based on literature data and on our pilot studies, we selected five genes with potential roles in human OP: alkaline phosphatase (ALPL), matrix metalloproteinase 2 (MMP2), tissue inhibitor of metalloproteases 2 (TIMP2), fibroblast growth factor receptor 1 (FGFR1), and fatty acid-binding protein 3 (FABP3). We aimed to investigate the effect of multiple SNPs in ALPL, FABP3, FGFR1, MMP2, and TIMP2 on OP in postmenopausal women.

Over 1 million vertebral compression fractures - mostly caused by osteoporosis - occur worldwide annually. Morbidity and mortality significantly increase in patients who suffered vertebral compression fractures, and the incidence of this type of fracture rises continually. Cement augmentation of the fractured vertebral body is a minimal surgical technique to reduce pain and kyphotic deformity, as well as to stabilize the affected segment. The most common complication of the vertebroplasty procedures is the cement leakage

from the cracked vertebral body. In rare cases bone cement gets into epidural or foraminal space causing serious consequences. Cement leakage into adjacent disc - when the filler material appears in the substance of nucleus pulposus - is much more common (in 5%–25% of all vertebroplasties) but less investigated. There are little evidence about the clinical and biological issues of this type of cement leakage.

Bone is the second most frequently transplanted tissue after the blood so ideal bone substitution technique as well as optimal bone graft are very much needed in clinical work. Gypsum (calcium sulfate dihydrate, $CaSO_4 * 2H_2O$) have been used as synthetic bone substitute with good degradation and biocompatibility proprieties for a long while. Hungarian experiences about the orthopedic application of gypsum were represented for us by Prof. Tibor Riskó, who had implanted gypsum into tuberculotic bone voids in some patients in the medium of the past century. He observed normal bone ingrowth on the postoperative X-rays and clinical findings were also good. Despite the good clinical results there are very little data about the biological and physiological background of the gypsum-implantation and this lack of scientific evidence can detain the spread of this synthetic bone graft. The cellular changes attached to the implantation of bone grafts as well as proprieties, factors improving the availability of bone substitutes are still unknown too.

AIMS

We have been focusing on the following scientific questions in our doctoral works:

1. Is there any significant association between the individual polymorphisms of the ALPL, FABP3, FGFR1, MMP2 and TIMP2 genes and the bone mineral density and non-vertebral fracture risk?

2. Is there any association between the haplotypes of the studied genes and the osteoporotic phenotypes?
3. Is there any gene-gene interaction among the studied genes significantly affecting the bone mineral density or the fracture risk?
4. Do the different vertebroplasty bone cements influence the viability and the proliferation of the nucleus pulposus cells?
5. Have the vertebroplasty cements got any significant effect on the gene expression profile of the nucleus pulposus cells?
6. Are the MC3T3-E1 preosteoblast cells able to adhere and proliferation on the gypsum surface?
7. How do the different synthetic bone substitutes and the high Ca^{2+} concentration affect the cell viability and the gene expression profile of the preosteoblast cells?

METHODS

Gene polymorphism studies

Three hundred sixty postmenopausal women were included into the study through our osteoporosis outpatient clinic. Subjects arrived to their first densitometry screening. BMD values were measured at lumbar spine (L2-L4) and total femur with a Lunar Prodigy DXA densitometer, at radius with a Norland pDEXA® densitometer. Detailed fracture history was recorded from subjects. The study was approved by the ethical board and written informed consent was obtained from all participants.

Twenty-four SNPs were chosen for genotyping in the studied five genes (ALPL, MMP2, TIMP2, FGFR1, FABP3) based on the data from NCBI dbSNP and HapMap databases. Genomic DNA was isolated from venous blood using High Pure PCR Template Purification kit. Robust genotyping was

performed at the SNPStream Core Facility of the Semmelweis University using a GenomeLab SNPstream® Genotyping high throughput System.

In vitro studies

Four young men operated with intervertebral disc protrusion were involved into the study. Nucleus pulposus (NP) cells were isolated from the surgically removed disc tissue by sequential enzymatic digestion. To determine the effects of vertebroplasty filler materials on isolated NP cells, we treated the cultures with polymetil-metacrilate (PMMA) particles, hydroxi-apatite (HA) and gypsum (gypsum) suspensions.

MC3T3-E1 preosteoblast cells were cultured on four different culture surfaces in previously prepared culture plates (TP). Gyspum surface was made by the suspension of $CaSO_4$ * $1/2H_2O$ and water, PMMA surface was made using extra low viscosity bone cement and cells were also cultured on sterilized mineral gypsum slices as well as on culture plate with elevated Ca^{2+} concentration to 25.5 mM in the medium.

Cell number of viable cells was determined with CellTiter-Glo® Luminescent Cell Viability Assay. Alkaline phosphatase activity and Ca^{2+} level in supernatant were measured using an Olympus AU2700 analyzer.

Total RNA was purificated from cell cultures and gene specific mRNA expression of more than 20 genes playing role in disc or bone metabolism was determined performing real time RT-PCR technique with the Applied Biosystem TaqMan® Gene Expression Assays system. “Housekeeping” gene of GAPDH was used for endogenous control in each case. Relative quantification data was collected by the 7500 System SDS software.

Statistical analysis

We used the “Haploview 3.0” software for the descriptive statistics of the genotyping. Genetic association analyses were performed using the

„PedGenie" software. To assess the reliability of the results, permutation procedures were performed to generate empirical p-values after 10 000 Monte Carlo permutations. Permuted global $p<0.05$ was considered significant. The statistical power of the study was calculated with the Quanto 1.1 software. We conducted haplotype analyses using the 'haplo.stats' software in the statistical environment 'R'. Gene–gene interactions (GxG) were tested using 'SNPassoc' software. This 'R' package, developed for genetic studies, determines GxG effects performing log-likelihood ratio tests (LRTs). To avoid false-positive results, only highly suggestive interactions characterized by a p-value less than 0.001 were selected and gene–gene interactions were validated under conditional regression models. Interactions with a $p<0.01$ in the regression model were considered significant. The regression analyses were performed using SPSS 15.0 for Windows.

In vitro results were also analyzed with the SPSS 15.0 for Windows software. In vitro data represented the results of at least four independent cultures In each experimental setup. Non-parametric Mann-Whitney U-test with 10000 Monte-Carlo permutations were performed for statistical comparisons. Permuted p-value less than 0.05 was considered significant. In gene expression studies, differences more than 2-fold were noted as positive results.

RESULTS

New gene polymorphisms associated with osteoporosis

rs6996321 in FGFR1 was significantly associated with lumbar BMD (adjusted mean BMD was -0.031±0.011 and 0.031±0.011 g/cm^2 in G/G and A/G+A/A genotypes, p=0.002). Homozygote recessive genotype of *rs10914367* in FABP3 was significantly related to higher femoral BMD (adjusted mean BMD was -0.004±0.008 and 0.143±0.037 in G/G+A/G and A/A genotype groups, p=0.028). We found a significant association between the non-vertebral

fracture risk and *rs9900912* variants of TIMP2. In carriers of the recessive allel ('A') fracture risk was two-fold higher than in non-carriers (p=0.018). Power of the study in cases of these associations was more than 80%.

Using the 'haplo.stats' software we identified a 4-loci haplotype in FGFR1 gene (*rs13317, rs3925, rs2280846 and rs6996321*) significantly associated with lumbar BMD (p_{global}= 0.007). Common TCGG haplotype yielded significantly low BMD while a less common one was proved to be a predictor of the high BMD.

Significant and validated gene-gene interactions were identified between the FABP3 and MMP2 (*rs10914367* and *rs1030868*) regarding the lumbar BMD. Interaction of ALPL and TIMP2 had significant influence on femoral (*rs3738099* and *rs9894295*) and radius (*rs871132* and *rs9894295*) BMD. The GxG interaction between the MMP2 and TIMP2 (*rs243847* and *rs931227*) was associated with the fracture risk.

Effect of vertebroplasty filler materials on viability and gene expression of nucleus pulposus cells

Number of viable cells was significantly decreased by PMMA based cement treatment in a dose-dependent manner. Cell-reducing effect of HA was also measured while gypsum affected the cell number only in case of the highest dose applied.

Effect of synthetic filler materials on cell number was also investigated regarding the treatment time. 0.1% PMMA resulted on 75.2 – 79 – 78.2 – 68.4 – 72% decrease in cell number in case of 1 – 2 – 3 – 4 – 5 day long treatment. 0.1% HA treatment yielded 40.2 – 46.5 – 51.5 – 53.5% reduced cell number depending on the 2 – 3 – 4 – 5 day long culture period. Gypsum treatment in 0.1% concentration did not affect significantly the cell number either in long treatment courses.

Expression of 12 gene was studied in isolated NP cells after 4-day long treatment of the filler materials. Expression of aggrecan (AGC1) was decreased

when VFMs were added to NP cell cultures (2.5-fold, 6.6-fold, 6.4-fold decrease with PMMA, CSC and CPC treatment). The reduction in AGC1 expression by PMMA was significantly different from that of CSC/CPC. We have seen a significant decrease in type I collagen (COL1A1) expression in PMMA- and CSC-cultures (PMMA: 2-fold, CSC: 4.7-fold decrease), The effect of CPC was not significant. We could not determine the effect of VFMs on type II collagen (COL2A1) expression because it was not expressed in vitro in detectable amounts. Biglycan (BGN) was expressed in reduced amounts after CSC and CPC treatment (5.4-fold and 8.7-fold), and these changes were also significantly different compared to PMMA treatment. (3.1-fold decrease with PMMA compared to CSC, 5-fold decrease with PMMA compared to CPC). mRNA of decorin (DCN) significantly decreased in CSC- and CPC-treated cultures of human NP cells (CSC: 2.5-fold, CPC: 2.5-fold decrease). Expression of fibronectin 1 (FN1) did not differ in treated cultures compared to untreated controls. There were some significant differences in the expression of matrix metalloproteinase 2 /gelatinase/ (MMP2), matrix metalloproteinase 13 /collagenase/ (MMP13) and tissue inhibitor of metalloproteases 2 (TIMP2) in NP cells due to VFM-treatment. MMP2 was expressed in the lowest amounts in CPC-cultures, and it was significantly different from MMP2 expression in PMMA-cultures (2.9-fold higher with PMMA). MMP13 was not detectable in CPC-cultures. Bone morphogenetic protein 2 (BMP2) was overexpressed after CSC or CPC treatment (4.1-fold and 9.3-fold increase compared to untreated controls as well as 2.6-fold and 6.63-fold increase compared to PMMA treatment). Expression of interleukin-1-alpha (IL-1A) significantly increased in CPC-cultures (5.3-fold) and was not detectable in CSC-cultures. SOX9 expression was decreased 3.3-fold after PMMA-treatment, while it was stimulated by CPC (5.8-fold increase). CSC did not change the expression of SOX9 compared to untreated cultures, but there were significant differences between the effects of CPC/CSC and PMMA (19.1-fold and 2.3-fold increase respectively).

Proliferation and gene expression profile of MC3T3-E1 preosteoblasts cultured on gypsum surface

MC3T3-E1 cells could be cultured on gypsum surface as well as in high Ca^{2+}-concentration. Cells were rather spindle-shaped on gypsum discs compared to culture plates where they were more cubical. MC3T3-E1 osteoblasts could not adhere to mineral gypsum slices. This spindle-shaped morphological change could also be noted on culture plates when medium was supplemented with Ca^{2+} to a final Ca^{2+} concentration of 25.5 mM. Degenerated and necrotic cells appeared in a high ratio on PMMA surface. Osteoblasts were viable on gypsum discs and they were able to proliferate on it with an increased ratio (1.89±0.091) compared to PMMA where cells were inhibited to grow (0.914±0.052) ($p<0.01$). Growth rate of MC3T3-E1 cells on gypsum discs were similar to plastic culture plates using medium supplemented with 25 mM of $CaCl_2$ (TP25). After 15 days of culture, alkaline phosphatase activity measured from supernatants was significantly higher on gypsum discs (23.14±4.55 U/g) and culture plates with Ca^{2+}-supplemented medium (30.07±3.04 U/g) than on culture plates with normal Ca^{2+} (4.44±0.52 U/g) and PMMA (4.08±0.62 U/g). SMAD3 expression in the different cell cultures has changed with the same tendency. Relative expression of this gene was 2.2-fold higher on gypsum than on PMMA ($p<0.01$).

A different gene expression profile was observed with quantitative real-time PCR on gypsum compared to culture plate with standard Ca^{2+}. Relative expression of type II collagen (COL2A1) was more than 130-fold higher ($p<0.001$) on gypsum surface. Expression of fibronectin 1 (FN1), SMAD3 and SMAD6 have also significantly increased in cells cultured on gypsum. Gene expression of type I collagen (COL1A1) was 12-fold increased ($p<0.001$) on culture plate with standard Ca^{2+}. Amount of gene specific mRNA of decorin (DCN) and bone morphogenic protein 4 (BMP4) were decreased on gypsum. Bone sialoprotein (BSP), osteocalcin (BGLAP) and calcium sensor (CASR) was not expressed in detectable amount on gypsum disc. When Ca^{2+} concentration

was increased in the standard medium to the extent of that present in the medium above gypsum discs, an 80-fold increase in COL2A1 and a 20-fold decrease in COL1A1 expression was seen in cultures with high Ca^{2+} level. SMAD6 and SMAD3 expressions also significantly increased due to Ca^{2+} supplementation, similarly to that seen in gypsum disc cultures. Amount of mRNA of DCN, BMP4 and BSP was decreased in the presence of high Ca2+ level. CASR and BGLAP have been expressed in cells cultured in standard α-MEM containing 1.8 mM Ca^{2+} but were not detectable in cultures with 25.5 mM extracellular Ca^{2+}. The expression profile in osteoblasts cultured in high Ca^{2+} medium was similar to that of cells grown above gypsum disc. MC3T3-E1 cells on gypsum disc expressed a large amount of COL2A1 compared to PMMA (51-fold difference, $p<0.001$), while expression of COL1A1 was 7-fold higher on PMMA. FN1, SMAD6 and SMAD3 were also overexpressed on gypsum disc but expression of BMP4 and DCN were significantly lower than on PMMA. CASR, BGLAP and BSP were not expressed on gypsum disc while detectable amount of gene specific mRNA of these genes was measured in cultures on PMMA. The expression profile in osteoblasts cultured on PMMA was similar to that of cells grown in culture plates with standard calcium concentration.

CONCLUSIONS

New associations described in our research work:

1. Studying the allelic variations of the new candidant genes described in the deer model, significant associations were found between the *rs6996321* SNP in FGFR1 gene and lumbar bone mineral density, between the *rs10914367* SNP in FABP3 gene and femoral BMD as well as *rs9900972* in TIMP2 and non-vertebral fracture risk.
2. In haplotype analyses we described the significant effect of the 4-locus haplotype of FGFR1 on lumbar BMD.

3. Highly suggestive gene-gene interactions among the five new candidant genes significantly related to BMD and fracture risk were first described in our study.
4. We confirmed in our work that the vertebroplasty filler materials had significant influence on viability and proliferation of isolated nucleus pulposus cells, underlying that gypsum based filler material proved to be more biologically compatible than the everyday used but toxic PMMA based cement.
5. Vertebroplasty filler materials significantly affected the gene expression profile of the isolated nucleus pulposus cells too. Final conclusion of our molecular study was that the intradiscal cement leakage during vertebral augmentation procedures could increase the risk for subsequent new vertebral compression fracture.
6. We described that preosteoblast cells could adhere, proliferate and produce matrix on gypsum based bone graft.
7. We published first that differentiation of preosteoblasts cultured on gypsum surface is was similar to bone healing phenotype which provides molecular evidence supporting the clinical use of gypsum as synthetic bone graft. Advantageous biological changes are related to the special crystal structure and high Ca^{2+}-content of the gypsum.

SUMMARY

Osteoporosis is a common disease where heritability plays also important role in the pathogenesis beyond the environmental factors but its complex genetic background is still unknown. Long-term results as well as biological considerations of the minimal invasive surgical treatment of the compression vertebral fractures are still less known. The molecular biological background of the surgical bone substitution as well as biological effects of synthetic bone grafts are also unclear topics of osteology. In our research work,

we identified new candidant genes and polymorphisms playing significant role in the process of osteoporosis. We also described that the most common complication of vertebral augmentation, the intradiscal cement leakage, could increase the subsequent new compression fractures and it could strongly depends on the type of the bone cement. We concluded that avoid of cement leakage and development of biologically and biomechanically optimal filler materials should be desired. We demonstrated the molecular biological background of the use of gypsum as synthetic bone graft as we first published that bone cells differentiated into the direction of new bone formation and this process was mediated by the high calcium content and special mineral structure of gypsum.

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