New Mechanism in the Regulation of Bone Metabolism

Doctoral thesis

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INTRODUCTION

Bone is a dynamic connective tissue, comprising an exquisite assembly of functionally distinct cell populations that are required to support the structural, biochemical integrity of bone and its central role in mineral homeostasis. The skeleton is a metabolically active organ that undergoes continuous remodeling throughout life. Bone remodeling involves the removal of mineralized bone by osteoclasts followed by the formation of bone matrix through the osteoblasts that subsequently become mineralized. Bone remodeling serves to adjust bone architecture to meet changing mechanical needs and it helps to repair microdamages in bone matrix preventing the accumulation of old bone. It also plays an important role in maintaining plasma calcium homeostasis. The regulation of bone remodeling is both systemic and local. The major systemic regulators include parathyroid hormone (PTH), calcitriol, and other hormones such as growth hormone, glucocorticoids, thyroid hormones, and sex hormones. Factors such as insulin-like growth factors (IGFs), prostaglandins, tumor growth factor-beta (TGF-beta), bone morphogenetic proteins (BMP), and cytokines are involved as well. As far as local regulation of bone remodeling is concerned, a large number of cytokines and growth factors that affect bone cell functions have been recently identified. Cross-talk between bone metabolism and immune system are now well described. Multiple soluble mediators (including cytokines, chemokines and growth factors) are also regulate the local interaction of osteoblasts / osteoclasts and immune cells in the common bone tissue microenvironment via overlapping signaling networks. The maintenance of bone involves a delicate balance between the actions of systemic hormones, local cytokines and growth factors, and physical forces. Disorders that interfere with, amplify, or mimic the effects of these factors can result in abnormal bone remodeling and elicit pathological conditions that result in increased susceptibility to fracture.

The aim of our investigation was to determine the role of systemic regulators (parathyroid hormone, thyroid hormones, and sex hormones) in bone metabolism at cellular , molecular and gene levels.

AIMS

I. Examination effect of PTH on bone

A potential role for PKC in resorptive processes is suggested by studies in which PTH-dependent resorption was decreased by PKC inhibitors and chronic cotreatment with phorbol esters**.**

I.A: To investigate the level at which protein kinase C (PKC) regulates expression of interleukin-6 (IL-6) in osteoblastic cells, effects of several PKC antagonists and PKC down-regulation by phorbol ester were studied in UMR-106 osteoblastic cells that had been transiently transfected with a −224/+11-base pair (bp) IL-6 promoter coupled to a luciferase reporter.and stimulated by of PTH, TNF-α, and IL-1β.

IB.: Furthermore we investigated, the importance of specific PKC isozymes in this process. A selective antagonist of PKC-beta, LY379196, was used to determine the role of the PKC-beta isozyme in the expression of IL-6 in UMR-106 rat osteoblastic cells and in bone resorption in fetal rat limb bone organ cultures. PTH, tumor necrosis factor-alpha (TNF-alpha), and interleukin-1 beta (IL-1 beta) induced translocation of PKC-alpha and -beta(I) to the plasma membrane in UMR-106 cells.

I.C: PKCα and PKCβI are translocated after treatment with PTH in UMR-106 osteoblastic cells; however, the pathway leading to PKC isozyme translocation is not established. Diacylglycerol (DAG) generation from phospholipase D (PLD) is one pathway of PKC activation, and PTH-mediated PLD activity is dependent on small G-proteins of the Rho family. This study investigated whether Rho proteins modulate the PKCα translocation and IL-6 promoter activity stimulated by PTH in UMR-106 osteoblasts.

II. Examination of polymorphisms of major candidate genes that may regulate bone metabolism in healthy and postmenopausal women.

Regulation of bone mass is a polygenic process and during recent years, studies have identified polymorphisms in several candidate genes that have been associated with bone mass or osteoporotic fractures, including vitamin D receptor (VDR) gene, estrogen receptor (ER) gene, and calcium-sensing receptor (CaSR) gene .

II.A: We investigate the impact of most common A986S polymorphism of the CaSR gene on bone mineral density in a population of postmenopausal osteoporotic women.

II.B: We investigated the impact of polymorphism of the IL-1RN gene on bone mineral density in a large population of Hungarian postmenopausal women.

III.C: In our study, we investigated a relationship between the BMD and the *Bsm*I polymorphism of the vitamin D receptor (VDR) gene in postmenopausal women.

III.D: We analysed the *Pvu*II and *Xba*I polymorphisms of the oestrogen receptor-α (ERα) gene in in hungarian healthy and osteoporotic women.

III. Effects of Estrogen on bone

III.A: The production of interleukin-6 (IL-6) by cultured bone marrow stromal and osteoblastic cell lines is inhibited by estrogens, although this is not a finding in all osteoblastic

cell preparations. The inhibitory effect of estrogen on IL-6 production is mediated by the inhibition of IL-6 gene transcription through an estrogen-receptor-mediated indirect effect on the transcritpional activity of the proximal 224-bp sequence of the promoter. The ERβ is highly homologous to the classical ERα, and activates genes containing an ERE in an estrogen-dependent manner. The ERβ binds to estrogens with an affinity slightly less than that of the ERα; the K_d difference between the two receptor types is 0.5 log. The ligand-binding affinities and DNA-binding domains of ERα and ERβ are similar, but their transactivation domains, tissue distribution, and molecular sizes differ significantly. In some cell systems, using transfection studies with reporter genes, the ERβ was shown to be a weaker regulator of gene transcription than ERα. However, the ERβ is more highly expressed in bone and may play a more prominent role in bone than ERα. The goal of this study was to compare the two human estrogen receptor types for their effects on the regulation of IL-6 gene expression.

III.B: Earlier experimental studies have investigated possible effects of estrogen on only one or two possible target genes in bone. The aim of this study was to search for possible mechanisms of action of estrogen on bone by studying the expression of several bone-related genes using the DNA microarray technique. A second aim was to determine the ER specificity for possible regulated genes**.**

III.C: Th**e** results of animal studys not directly connected, or not easily transformabile for humans. Therefore aim of our investigation was to determine genes characterized by significantly changed mRNA expression rates in postmenopausal *vs.* premenopausal non-osteoporotic bone tissue applying Taqman probe based quantitative real time PCR as well as to describe the interrelationships among these genes using multivariate data analysis.

IV. **Effects of thyroid hormones on bone.**

Thyroid hormones have both anabolic and catabolic effects on bone. Anabolic effects, which are important for normal growth and the maintenance of bone tissue integrity, may be mediated through insulin-like growth factors . CAtabolic effects of thyroid hormones on bone tissue might be mediated by IL-6. The mechanism by which thyroid hormones promote bone growth and loss has not yet been elucidated.

IV.A: The aim of this study was to determinate the effect of PTH, oestrogen and thyroid hormones on the regulation of IL-6 gene expression.

IV.B:. To determine whether the IGF-I/IGF binding protein (IGFBP) profile is affected when thyroid hormone production is altered in vivo, we studied 36 women who had recently been diagnosed with hyperthyroidism (age: 29-67 years; 19 with Graves' disease, 17 with toxic nodular goiter) and 36 age-matched healthy women as controls

IV.C: We have studied serum TNF-α levels, TNF-α production by mononuclear cells, and bone mineral content in patient with thyroid disorders.

III. MATERIALS AND METHODS

3.1. Cellbiological methods

3.1.1. Mononuclear cell cultures: Mononuclear cells were separeted on Ficoll/Hypaque, were resuspended in medium at a concentration of 10^6 cells/mL and 1 mL aliquats were placed in a 24 well tissue culture plate for 2 h. at 37°C, in a humidified atmosphere containing 5 % CO_2/95 % air. The adherent cells were then incubated for 48 h. in 1 mL RPMI/1640 suplemented with 10 % heat-inactivated FBS ± 1 μg/mL Phytohemagglutinin . Supernatants from quadruplicate cultures were stored at -20°C until asseyed.

3.1.2 Cell culture: UMR-106 osteoblastic osteosarcoma cells were maintained in DMEM supplemented with 15% heat-inactivated horse serum and 100 U/ml K-penicillin G at 37°C in a 5% CO2 environment. Cells were passaged every 3-5 days in 75-cm2 tissue culture flasks. Cells were used for experiments between passages 1-19. In same experiment we are used MC3T3, ROS, MG123, L929 cell lines. Mause bone marrow culture:Bone marrow from new born (5-7 d.) mice was purified and centrifuged. Collected cells were cultured for 6-8 days in MEM+15%HIFCS with 1,25 D3 vitamin.

3.1.3. Biochemical measurements: Blood was drawn after an overnight fast, centrifuged, and serum was stored at -20°C until determination. TNF-α bioactivity was detected by using L929 cell citotoxicity assay . In the bioassay recombinant human TNF-α was applied as a standard in a concentration range of 0,625 pg/ml – 1,562 pg/ml. Serum TNF-α bioactivity was expressed in units, one unit corresponding to the bioactivity of the standard caused 50% of citotoxicity (25 pg of standard TNF-α/well). Monoclonal neutralizing anti TNF-∝ and β mouse immunoglobulin were used for the verification of the presence of TNF-∝ or β bioaktivity of samples. Each determination was carried out in quadruplicate. The sensitivity of the method was under 1 pg/mL.

3.2. Human and animal experiments

3.2.1.Subjets**:** Hungarian unrelated women were recruited to the studys through our clinic. Subjets in the age range 40-90 years were recruited**.** Exclusion criteria were history of bone, metabolic, or endocrine disease; any chronic illness; hormone replacement, steroid therapy, or any medication known to influence bone metabolism. Subjects with biochemical abnormalities such as increased levels of serum ALPL, thyrotrophin, parathyroid hormone, or reduced level of 25-OH vitamin D (<30 ng/ml) were not included in the study. There were no difference in body mass index, smoking habits, calcium intake, and alcohol and caffeine

consumption between the OP and C groups. The studys were approved by the local ethics committee and all petients gave written informed consent.

3.2.2. Human bone tissue samples: Gene expression profile in bone samples was determined in postmenopausal, unrelated, consecutive, Hungarian, Caucasian non-osteoporotic women (POST group) who did not received hormonal replacement therapy. Menopause was defined according to the recent WHO criteria (at least one year amenorrhea, retrospectively) and serum estradiol level less than 30 pg/ml. The control group included bone tissue samples from premenopausal non-osteoporotic women (PRE group). There were no significant differences between the pre- and postmenopausal non-osteoporotic groups in age, bone mineral density, smoking habits, calcium intake, alcohol, caffeine consumption and physical activity, and they have not received any steroid or biological therapies either. Bone tissue samples were collected from the spongious substance of femoral head during total hip arthroplasty before resection. Surgically removed bone samples were extensively washed in PBS for eliminating blood, fat and marrow contamination and placed immediately into liquid nitrogen. The study was approved by the Regional Committee of Science and Research Ethics, Semmelweis University (6392-1/2004-1018EKU) and all patients gave written informed consent.

3.2.3.: Mouse bone tissue samples:Three groups of eighteen five-week-old female CD-1 mice were studied. Two groups were ovariectomized (OVX) and the third sham operated group served as normal control group (CTR). After recovery, one group of OVX mice (estrogen replacement-treated = ERT) was injected with 250 µl 30µg/body weightkilogramm 17β-estradiol-3-benzoate dissolved in sesame oil, subcutaneously three times a week. Control mice received injections of vehicle. The plasma estradiol levels were measured after the injection in several time points, and they were close to the average plasma levels of estradiol in the control animals. (plasma estradiol: CTR: 67, 3±8, 2 pg/ml, OVX: 1, 0±1, 9 pg/ml, ERT: 76, 3±16.6 pg/ml; no significant difference between CTR and ERT). The mice were sacrificed after two weeks, and the femurs and tibiae were dissected. The bones were immediately placed in liquid nitrogen after removal. The study was approved by the local Animal Care Committee.

3.3. Molecular biology methods

3.3.1. Plasmid construction : Human IL-6 promoter consisted of base pairs −779/+11, −224/+11, −158/+11, −109/+11, and −49/+11 from the promoter region of the gene. The vector was a modified thymidine kinase-chloramphenicol acetyltransferase (TK-CAT) vector, in which TK was replaced by the IL-6 promoter. For the current studies, the promoter construct was coupled with a luciferase reporter. The Luc gene was from GL2-Basic. The

*Xho*I/*Hpa*I Luc fragment from GL2-Basic was cloned into *Xho*I/*Hpa*I-digested IL-6-CAT.

The $ERE_2tk_{109}luc$ reporter construct consists of a double vitellogenin ERE with a basal thymidine kinase promoter and a luciferase gene. Human ERα cDNA (HEGO) construct was subcloned into pcDNA3.1(-) using *EcoRI* sites. Human ERβ was cloned from a normal human testis cDNA library using published rat ERβ sequence and degenerate primers. The cDNA clone was sequenced and matched to the published human ERβ sequence in reported in Enmark et al.. The cDNA construct was subcloned into pcDNA3.1(+) using *AvrII* and *NotI* sites. Rat ERα cDNA construct was subcloned into pcDNA3.1(-) using *EcoRI* sites. The rat ERβ cDNA plasmid was cloned from a rat prostate cDNA library using published sequences, and subcloned into pcDNA3.1(-) using *ApaI* and *NotI* sites. Human IGF-I promoter costruct consisted of base pairs -4770, -1208, -1033, -834, -506, -450, -184 and –126 from the promoter region of the gene. The rat IGF-I promoter -450 bp region. TRE-Luc promoter, the human thyroid response element and luciferase gene.

3.3.2.Transient transfections, Luciferase assay: UMR-106 cells were plated on 22-mm2glass coverslips or at 75,000 cells per coverslip. After overnight incubation, cells were transfected with empty vector (pcDNA3), constitutively active RhoA (RhoA63L), or dominant negative RhoA (RhoA19N). Transfections were performed using Lipofectamine Plus (Invitrogen/Gibco Life Technologies). For each transfection, 1 μg DNA, 2 μl Plus Reagent, and 97 μl OPTI-MEM were used, followed by a 15-minute incubation with 1 μl Lipofectamine in 99 μl OPTI-MEM. Complexes were added to cells for 3 h, followed by addition of 1 ml OPTI-MEM + 5% heat-inactivated FBS and penicillin/streptomycin. Transfected cells showed normal cell viability by MTT assay. After 24 h, transfected cells were treated, fixed, permeabilized, and stained for PKC isozymes. For IL-6 promoter transfections, cells were plated at 75,000/well in a 24-well plate. Cells were transfected with a −224/+11-bp fragment of the IL-6 promoter upstream of a luciferase reporter. Approximately 400 ng of DNA was used with 2 μl PLUS and 1 μl Lipofectamine in 200 μl OPTI-MEM. Luciferase activity was measured 24 h after transfection. Treatments were added the last 5 h of the assay. Media were aspirated, and extraction buffer (25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 1% vol/vol Triton X-100, and 2 mM dithiothreitol [DTT]) was added. After 15 minutes, the assay mix buffer (25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 2 mM DTT, 15 mM K2HPO4, pH 7.8, and 20 mM ATP) was added to each well. The entire cell lysate was added to tubes, and luciferase activity was determined after the addition of the luciferin cocktail (25 mM glycylglycine, pH 7.8, 0.2 mM luciferin, and 10 mM DTT). Luciferase activity was measured using a Monolight 2010 luminometer.

Relative light units (RLUs) represent luciferase activity and are a measure of arbitrary light units per 10-s interval, after subtraction of a reagent blank.

3.3.3:_PKC Assay: UMR-106 cells were plated in 100 mm petri dishes with DMEM + 5% heat -inactivated FBS + penicillin/streptomycin. After overnight incubation they were 80%-90% confluent. Treatments were added for the last 5 hr of the assay. Following treatment, the medium was removed and the petri dishes washed twice with PBS at room temperature. Cells were lysed in RIPA buffer (1x PBS, 1% NP40. 0.5% sodium deoxycholate, 0.1% SDS) containing 10 mg/ml PMSF, 30 μg/ml aprotinin, 100 mM sodium orthovanodate. The protein content of cell lysate was measured with a Coomassie Plus protein assay kit. PKC was detected with the non-radioactive PepTaq^{TM} assay kit.

3.3.4: Subcellular fractionation: Cells were cultured in 75-cm2 tissue culture flasks until subconfluent. Cells were then plated at 1:10 in 100 × 20 mm2-tissue culture dishes and grown to subconfluence, which was ~48 h. Cells were serum-starved for 24 h before the experiment. Cells were treated for the indicated times with PTH(1-34) or PTH(1-34) at the indicated concentrations. Cells were washed with 5 ml HBSS once, and 500 μl of homogenization buffer (12.5 mM Tris HCl, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 5 mM DTT, pH 7.4) containing 0.05% digitonin and protease inhibitors (0.26 mg/ml phenylmethylsulfonyl fluoride [PMSF], 0.15-0.3 TIU/ml aprotinin, 5 mM sodium orthovanadate, and 0.1 mg/ml leupeptin) was added. Cells were scraped and homogenized by passage through a 21-gauge needle. After centrifugation of the homogenate at 14,000 rpm (18,000g), at 4°C for 15 minutes, cytosolic fractions were collected. The pellet was resuspended in 500 μl homogenization buffer containing 0.1%Triton X-100 and was centrifuged at 14,000 rpm (18,000g), at 4°C for 15 minutes. The supernatants were collected and designated membrane fractions. Sample buffer (17% glycerol, 8.7% [vol/vol] β-mercaptoethanol, 5% SDS, 0.2 M Tris-HCl [pH 6.7], and 0.1 mg/ml bromphenol blue) was added to the membrane fractions, which were boiled for 5 minutes. Protein concentration was determined on an aliquot of the lysates using the Lowry assay, and equal amounts of protein were loaded per well for SDS-PAGE.

3.3.5: Western blotting: Membrane fractions or whole cell lysates (50 μg) were subjected to SDS-PAGE on 12.5% acrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes. To confirm that the wells were evenly loaded and that the transfer was successful, the membranes were stained with Ponceau S. The membranes were incubated with blocking buffer containing 2% bovine serum albumin (BSA) and 2% evaporated milk in Tris buffered saline plus 0.05% Tween (TBS-T) for 1 h. Primary antibodies diluted in blocking buffer were added to the membranes overnight. Anti-RhoA was used at 1:1000 in

blocking buffer. After two washes with TBS-T, the membranes were incubated in an anti-rabbit or anti-mouse IgG-HRP secondary antibody at 1:2000 for 30 minutes. Immune complexes were visualized using enhanced chemiluminescence.

3.3.6: Rhotekin assay : UMR-106 cells were grown to subconfluence on 10-cm plates and serum-starved overnight. Cells were treated with vehicle, 10 nM PTH(1-34), or 10 nM PTH(1-34) for 3.5 minutes at 37°C before lysis in lysis buffer (50 mM Tris HCl, pH 7.4, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF, 100 mM NaCl, 5 mM MgCl2, 10 mg/ml aprotinin, and 10 mg/ml leupeptin). Cells were scraped, transferred to microcentifuge tubes, and clarified by centrifugation. Lysates were incubated with sepharose-bound GST-Rhotekin Rho binding domain beads for 45 minutes at 4°C. The lysates were centrifuged, and the beads were washed four times with lysis buffer. Proteins were eluted by addition of Laemmli buffer and boiling for 5 minutes. SDS-PAGE was performed as described above, and precipitated GTP-bound RhoA was normalized to total RhoA in the lysates.

3.3.7: Immunofluorescence : UMR-106 cells were seeded on 22-mm2 glass coverslips at 375,000 cells per coverslip. When cells were ~50% confluent, cells were treated with PTH(1-34), PTH(1-34), C. difficile toxin B, Y27632, phorbol 12,13 dibutyrate, or combinations for the indicated times and concentrations. Cells were fixed and permeabilized using ice-cold methanol:acetone (1:1), followed by three washes in PBS. Fixed cells were stained with a PKCα (1:100) isozyme-specific anti-PKC antibody and FITC-conjugated secondary antibodies (1:50). Cells were visualized with a Zeiss LSM 510 laser scanning/confocal microscope with a 63× oil immersion objective or with an Olympus Fluoview confocal microscope using a 60× oil immersion objective. Each experiment was performed three times. Within each experiment, six images per treatment were obtained in a blinded fashion. Each image was analyzed for the number of cells with membrane staining. The criteria for membrane staining was the presence of intense fluorescence on all sides of the cell perimeter.

3.3.8. RNA, DNA preparation: Total RNA was purified from each group (100-125 mg tissue from each animal) by modified TRIzol method. Briefly, bone samples were cleaned of soft tissues and homogenized in 1 ml TRIzol Reagent per 50-100 mg of tissue using glass-Teflon and liquid nitrogen. After incubating homogenized samples for 5 minutes at room temperature, 0,2 ml of phenol/chloroform per 1 ml of TRIzol Reagent was added and samples were centrifuged at 4 °C at 10 000 RPM for 15 sec. For precipitation of RNA, 0,5 ml isopropyl alcohol per 1 ml of TRIzol Reagent was used. After centrifugation at 4 °C at 10 000 RPM for 15 sec, RNA pellet was washed with 75% ethanol. Samples were further purified with SV Total RNA Isolation kit according to the manufacturer's instructions. The quantity and quality of RNA from each sample were assessed by gel electrophoresis as well as

spectrophotometry. RNA within a given group was pooled and used in replica experiments. Total RNA was used for microarray analysis as well as for reverse transcription quantitative PCR. Human bone samples (approximately 500 mg) were cryo-grinded under liquid nitrogen using a freezer-mill 6750. Direct mRNA isolation using Dynabeads Oligo $(dt)_{25}$ kit according to manufacturer's protocol. After DNase treatment mRNA was cleaned with NucleoSpin RNA Clean-up kit. The quantity and quality of purified mRNA were checked by NanoDrop spectrofotometer at 260/280 nm. Using 200 U SuperScriptIII RNase H - Reverse Transcriptase, 100 ng of each human mRNA sample was reverse transcribed. Genomic DNA was isolated from EDTA blood using a commercially available kit. The quantity and quality of purified mRNA were checked by NanoDrop spectrofotometer at 260/280 nm.

3.3.9. Microarrays and probes: 3200 amplified cDNA inserts from different mouse cDNA libraries were amplified with vector-specific primers, analyzed with agarose gel-electrophoresis, purified with Millipore PCR purification plates and reconstituted in 50% DMSO/water at a final concentration of 0.8 mg/ml. Purified PCR products were arrayed in duplicate on amino-silanized slides using a MicroGrid Total Array System spotter with 16 pins in a 4x4 grid format. After printing, DNA was UV crosslinked to the slides with 700 mJ energy. Prior to hybridization, the slides were blocked in 1 x SSC, 0.2% SDS, 1% BSA for 30 min at 42 ^{o}C, rinsed in water and dried. 2 μg total RNA from each pooled sample was amplified. 2 μg of amplified RNA was labeled with Cy5 or Cy3 fluorescent dyes during reverse transcription (RT) with RNAse H (-) point mutant M-MLV reverse transcriptase and random nonamers. After RT, RNA was alkali hydrolyzed and labeled cDNA was purified with NucleoSpin PCR purification kit according to the manufacturer's instructions. Probes generated from the control and treated bone samples were mixed, reconstituted in 12 μl hybridization buffer (50 % formamide, 5x SSC, 0.1 % SDS, 100 mg/ml salmon sperm DNA) and applied onto the array after denaturation by heating for 1 min at 90ºC. The slide was covered by a 22 mm x 22 mm coverslip, and sealed with DPX in order to prevent evaporation. Slides were incubated at 42ºC for 20 hours in a humid hybridization chamber. After hybridization, the mountant was removed and the arrays were washed by submersion and agitation for 10 min in 1 x SSC with 0.1% SDS, for 10 min in 0.1% x SSC with 0.1% SDS and for 10 min in 0.1 x SSC at room temperature, then rinsed briefly in deionized water and dried.

3.3.9. Scanning and data analysis: Each array was scanned under a green laser (543 nm, laser power: 95%, gain: 70%) or a red laser (642 nm, laser power 95%, gain: 75%) using a ScanArray Lite scanning confocal fluorescent scanner with 10 μm resolution. Image analysis was performed by the GenePix Pro 3.0 software . Each spot was defined by automatic

positioning of a grid of circles over the image. The average and median pixel intensity ratios calculated from both channels and the local background of each spot were determined. This average expression ratio for all genes on the array was normalized to 1.0. For background corrections those data were calculated as negatives where the percentage of pixels having fluorescent intensity over background plus 2x standard deviation was below 60 % (%>B649+2SD and %>B550+2SD) in either channel. Each experiment was performed twice using both fluorescent dyes for labeling control and sample to reduce the number of false positive or false negative ratios deriving from possible uneven incorporation of fluorescent dyes during labeling or from other experimental variables introduced by hybridization, washing conditions or array features. Therefore, from each RNA pool, two probes were generated: a Cy5-labeled and a Cy3-labeled one in order to perform replicate "color-flip" experiments suggested by other authors (10-14). Replica spots (on the same array) and replica experiments (two different arrays) resulted in four data points for every gene. Those spots were excluded from further analysis, where ratios of the replica spots have more than 2-fold differences. The same criteria were applied for definition of regulated genes considering the average-fold change (increase or decrease) of genes from the replica experiments. The expression ratios of genes were determined by calculating the fold change in average between OVX/CTR and ERT/CTR groups. The average-fold increase or decrease of the comparisons should be at least 2-fold to be considered as significant intensity ratio.

3.3.10. Real-time quantitative fluorescent reverse transcription: QRT-PCR was performed on a RotorGene 2000 instrument with gene-specific primers and SybrGreen protocol to confirm the gene expression changes observed by using microarrays. They were purchased from Avidin Ltd. 5 μg of total RNA from each pool was reverse transcribed in the presence of poly (dT) sequences in total volume of 20 μl. After dilution of the mix with 80 μl of water, 2 μl of this mix was used as template in the QRT-PCR. Reactions were performed in a total volume of 20 μl (2 pmol/each forward and reverse primers in 1X BioRad SYBRGreen buffer, with the following protocol: 10 minutes denaturation at 95 ^{o}C, and 45 cycles of 25 sec denaturation at 95 ^{o}C, 25 sec annealing at 59 ^{o}C and 25 sec extension at 72 ^{o}C. Fluorescent signals were collected after each extension step at 72 ^{o}C. For quantitative real-time RT-PCR comparison of gene expression patterns of candidate genes in the bone tissue of postmenopausal and premenopausal non-osteoporotic women, we selected 147 genes based on recent literature, OMIM database, as well as our data concerning genetic pathway analysis. Predesigned and validated gene-specific TaqMan Gene Expression Assays from Applied Biosystem were used in triplicate for quantitative real-time PCR according to the manufacturer's protocol.

3.3.11. Genotyping: Genomic DNA was extracted from peripheral blood by a commercially

available. DNA was subjected to polymerase chain reaction amplification using specific primers. The BsmI restriction site is missing in the B allele and it is present in the b allele. The lack of the XbaI/PvuII restriction sites corresponds to the X/P alleles and they are present in the x/p alleles. For the investigation of A986S polymorphism of the CaSR gene, , A and S alleles were recognized. All PCR reactions were carried out in Hybaid Express thermocycler .

3.4. Biochemical methods: Serum IGF-I, and its binding proteins (IGFBP-3, IGFBP-4, and IGFBP-5) were measured using a Nichols Institute RIA kit. The immunoreaktive TNF-α concentration was detected by using a commercial radioimmunoassay kit The sensitivity of the metod was 10 pg/mL. Blood serum TSH, fT3, fT4 levels where measured with commercial RIA.

3.5. Bone densitometry: Bone density was measured at the radius midshaft by single photon absorptiometry , and at the lumbar spine (L2-L4) and the femoral neck by dual X-ray absorptiometry . The bone mineral density (BMD) at the lumbar spine and femoral neck was expressed as an area density in grams per square centimeter while at the radius midshaft in grams per centimeter. For the purpose of the WHO classification, BMD was also expressed as t-score, which relates actual BMD values to healthy young population mean. Osteoporosis was diagnosed if the BMD value was below –2,5 t-score.

3.6. Multiparametric statistical analysis

We used standardized PCA (i.e., analysis from a correlation matrix between genes) because there were still excessive differences in the variance of variables. In the biplot graphic drawn for two components at a time, the observations (in this case, the subjects) appear as points, whereas the variables (in this case, the genes) are emphasized by lines pointing to their positions. This simultaneous representation allows for the evaluation of the grouping of subjects and of the relative importance and correlations of genes in influencing this configuration. Groups may be further illuminated by superimposing convex hulls on the ordination plane. Whereas PCA explores the total variance in the data, canonical variates analysis (CVA, alias discriminant function analysis) maximizes separation of *a priori* defined groups of observations. The results of CVA are canonical scores obtained from the canonical functions derived through eigenanalysis, which serve as coordinates of observations in the canonical space. Since the number of canonical axes is one less than the number of groups, in our case CVA produced only one variate. For the transfections studys values are expressed as mean ±SEM. Statistical significance was determined by Analysis of Variance (ANOVA) with Bonferroni's multiple-comparison post-test. The probability value of $p \leq 0.05$ was used to indicate statistical significance. Most experiments were repeated more than three times, and all treatments were carried out at least in triplicates. For QRT-PCR statistics curves were

analyzed using dynamic tube and slope correction methods with ignoring data from cycles close to baseline. Relative expression ratios were normalized to β actin. Results were calculated with delta-delta Ct method. All the PCRs were performed four times in two separate runs, and average values, standard deviations and p-values were calculated by using Student t-test. Estrogen effect was considered to be confirmed if OVX/CTR was significant, ERT/CTR was not significant, and the relative QRT-PCR expression ratio was greater than 2-fold in OVX/CTR relation. In those cases where estrogen replacement changed the expression ratio to the opposite direction as in ovariectomy but it did not return to the CTR level (i.e. it remained greater than 2-fold), estrogen effect was taken as confirmed if the difference between OVX/CTR and ERT/CTR expression ratios was significant. For the genotyping studys we using Student t test with separate variance estimates.

IV. RESULTS

I. Effect of PTH on bone

Parathyroid hormone (PTH) elicited a dose-dependent stimulation of the IL-6 promoter expression, with significant increases produced by 5 h of treatment with concentrations of PTH as low as 10(-14) M. The increase in IL-6 promoter expression was inhibited by the PKC antagonists GF109203X, 30 nM to 1 microM, and calphostin C, 250 nM. Prior down-regulation of PKC with 100 nM phorbol-12,13-dibutyrate (PDBU) for 48 h inhibited the PTH effect as well as the smaller stimulatory effects elicited by tumor necrosis factor alpha (TNF-alpha), 10(-9)-10(-8) M, and by IL-1beta, 1-10 ng/ml. In contrast to these findings, the stimulatory effects of PTH, TNF-alpha, and IL-1beta on the IL-6 promoter expression were enhanced by staurosporine. Treatment with GF109203X or down-regulation of PKC with PDBU prevented the stimulatory effects of staurosporine. PKC activity was increased by staurosporine. The findings with staurosporine are consistent with our earlier observations that this agent enhances the calcium signaling and bone resorption elicited by PTH. Further evidence that staurosporine was activating PKC in the cells was obtained with a direct assay of PKC activity . The studies support the role of PKC in the stimulatory effects of PTH, TNF-alpha, and IL-1beta on IL-6 expression.. Protein kinase C (PKC) has been shown to be activated by parathyroid hormone (PTH) in osteoblasts. Prior evidence suggests that this activation mediates responses leading to bone resorption, including production of the osteoclastogenic cytokine interleukin-6 (IL-6). However, the importance of specific PKC isozymes in this process has not been investigated. A selective antagonist of PKC-beta, LY379196, was used to determine the role of the PKC-beta isozyme in the expression of IL-6 in UMR-106 rat osteoblastic cells and in bone resorption in fetal rat limb bone organ cultures. PTH, tumor necrosis factor-alpha (TNF-alpha), and interleukin-1 beta (IL-1 beta) induced

translocation of PKC-alpha and -beta(I) to the plasma membrane in UMR-106 cells within 5 min. The stimulation of PKC-beta(I) translocation by PTH, TNF-alpha or IL-1 beta was inhibited by LY379196. In contrast, LY379196 did not affect PTH, TNF-alpha-, or IL-1 beta-stimulated translocation of PKC-alpha. PTH, TNF-alpha, and IL-1 beta increased luciferase expression in UMR-106 cells transiently transfected with a -224/+11 bp IL-6 promoter-driven reporter construct. The IL-6 responses were also attenuated by treatment with LY379196. Furthermore, LY379196 inhibited bone resorption elicited by PTH in fetal rat bone organ cultures. These results indicate that PKC-beta(I) is a component of the signaling pathway that mediates PTH-, TNF-alpha-, and IL-1 beta-stimulated IL-6 expression and PTH-stimulated bone resorption. The role of small G-proteins in PTH-stimulated PKC translocation and IL-6 promoter expression in UMR-106 cells was determined. The effects of PTH(1-34) and PTH(3-34) in stimulating PKCalpha translocation and IL-6 were inhibited by agents that interfere with the activity of small G-proteins of the Rho family and with the downstream kinase Rho kinase. Inhibition of Rho proteins with Clostridium difficile toxin B or inhibition of Rho prenylation with GGTI attenuated PTH(1-34)- and PTH(3-34)-stimulated translocation of endogenous PKCalpha and IL-6 promoter activity. Expression of a constitutively active RhoA (RhoA63L) mimicked the effect of PTH(1-34) or PTH(3-34) to promote membrane localization of PKCalpha, whereas cells expressing a dominant negative RhoA (RhoA19N) did not respond to PTH(1-34) or PTH(3-34). The Rho kinase inhibitor Y27632 attenuated PTH(1-34)- and PTH(3-34)-stimulated PKCalpha translocation and IL-6 promoter activation. Rho seemed to be acting at a step before production of diacylglycerol (DAG), because the stimulation of PKCalpha translocation by the DAG mimetic phorbol 12,13 dibutyrate (PDBu) was unaffected by C. difficile toxin B or Y27632. These results indicate that Rho proteins are an important component of PTH signaling in osteoblastic cells and provide further demonstration of convergence between PKC and small G-protein signaling pathways.

II. Effect of polymorphisms of major candidate genes on the bone metabolism in healthy and postmenopausal women.

We found a significant correlation between the VDR gene BsmI polymorphism and the femoral neck BMD. ($p<0.05$). We also found, that the number of subjects with BB genotype significantly higher is the hungarian population. The polymorphisms of the XbaI and PvuII genotype distributions of the ER alpha gene has no significant effect on BMD or biochemical parameters of bone turnover was observed. There was no correlation between IL-1RN alleles or genotypes and BMD in the 286 subjects. Dividing subjects into osteoporotic and healthy groups (osteoporotics and controls), we found no difference in the distribution of alleles or

genotypes between groups. We found no association between IL-1RN alleles or genotypes and BMD either at the lumbar spine or the femoral neck within groups. Our data do not support the hypothesis that this IL-1RN gene VNTR polymorphism has an impact on bone mass in postmenopausal women. We found also no difference in the distribution of different A986S polymorphisms of the CaSR gene alleles or genotypes between groups ($p = 0.762$). No significant effect of CaSR genotype on BMD was observed either in the whole population or in the subgroups. Our data do not support the idea that CaSR gene A986S polymorphism has an impact on bone mass.

III. Effects of Estrogen on bone

In UMR-106 osteoblastic cells transfected with the vitellogenin ERE-reporter construct, 17β-estradiol had similar positive regulatory effects in the cells transfected with either ERα or ERβ. In contrast, in UMR-106 cells transfected with the –224/+11bp human IL-6 promoter reporter construct, E2 was a more effective inhibitor of PTH-stimulated expression of IL-6 in the cells transfected with ERα than in cells transfected with ERβ. Similar results were obtained when 10x more ERβ than ERα expression plasmid was used.16α-Bromoestradiol inhibited PTH-stimulated IL-6 promoter expression in cells transfected with ERα, whereas it failed to affect the PTH response in cells transfected with ERβ. Genistein did not significantly affect the PTH-stimulated IL-6 promoter activity in cells transfected with ERα, but inhibited in those transfected with ERβ. Rat isoforms of the nuclear receptor were cloned and shown to have similar properties to the human receptors in the rodent UMR-106 cells on induction of the ERE reporter construct, inhibition of PTH-stimulated IL-6 expression and response to isoform- specific ligands. The results demonstrate that the E2-ERα complex represses IL-6 gene activation mediated by PTH more efficiently than does E2-ERβ. Further we examined the expression profiles of over 3,200 genes in the femurs and tibiae of sham operated (CTR), ovariectomized (OVX), and ovariectomized plus estrogen-replaced mice (ERT). Using strict analysis criteria, 1038 genes showed significant intensity ratios and 6.7 % showed altered expression: 30 genes exhibited significant up-regulation and 28 were down-regulated. We focused on those genes that showed alterations in their expression patterns both in OVX and ERT mice. Altogether 905 data points could be evaluated, from which 5.2 % showed altered expression: 23 genes exhibited significant up-regulation and 23 had down-regulation. In order to confirm the differential expression of genes revealed by microarray analysis of mouse bones after OVX with and without estrogen treatment, 12 genes were analyzed by QRT-PCR. We have identified genes such as L1cam, and non-muscle tropomyosin-5, cathelin-like protein, heparin cofactor II, protein synthesis elongation factor

Tu, and small GTP-binding protein Rab1a that have not been reported to have bone-specific regulation of their transcription. We have also shown genes that have been reported to be expressed in bone but have not been reported to participate in the mediation of estrogen action on bone. By using the microarray technique, we have found novel estrogen-regulated genes of potential importance for the bone-sparing effect of estrogen in mouse bone. In postmenopausal non-osteoporotic women, seven collagen molecules (COL2A1, COL3A1, COL5A1, COL5A2, COL9A1, COL12A1, COL15A1), three non-collagen type extracellular matrix (ECM) molecules (MGP, BGLAP, FN1) and two matrix degrading enzymes (MMP13, BMP1) showed significantly up-regulated expression patterns. Five genes belonging to the common TGFB/BMP signal transduction network (BMPR1A, TGFB2, TGFB3, TGFBR2, SMAD4) have been characterized by enhanced gene transcription rates in estrogen deficient women. The two indispensable transcription factors of osteoblasts (RUNX2, SP7) and the remaining transcription factors involved in the Wingless pathway (TCF7L2) and a chondrocyte maturation gene (SOX9) have shown increased expression in postmenopausal women compared to premenopausal ones. Two growth factors involved in MAPK signaling (PDGFA, FGFR1) also exerted overexpression in the absence of estrogen. Two osteoclast stimulating factors (TNFSF11/RANKL, IL6) and a main marker of differentiated osteoblasts (ALPL) had elevated expression activity in postmenopausal non-osteoporotic subjects.

IGSF4 and TRIB2 have been detected in non-osteoporotic human bone tissue for the first time. These genes were found to be over expressed in menopause. In case of ENO1/MBP1, diminished expression levels were observed. CVA was used to check whether the groups of post- and premenopausal women are separable in the multidimensional space spanned by the genetic variables, and if so, which gene subsets have the best discriminatory power. Nine gene subsets have been evaluated by CVA . Genes controlled via ER-beta exhibited the best discriminatory power which separates the postmenopausal and premenopausal non-osteoporotic subjects unambiguously. Genes coding ECM proteins and genes belonging to TGFB/activin/nodal pathway also showed strong correlation with the single canonical variate, hence achieving clear separation of the two groups of the examined non-osteoporotic women. The subsets of growth factors and BMP cascade had weaker discriminatory power .

V. **Effects of thyroid hormones on bone.**

We have analyzed IGF-I promoter activity using a series of human IGF-I promoter-luciferasefusions in UMR-106 cells (Figure 2). Gene fusions included human IGF-I promoter regions from -4770, -1208, -1033, -834, -506, -450, -184, and -126, and rat IGF-I promoter region -450 basepairs with respect to the ATG translation start site.These results suggest that T3 does not directlyregulated IGF-I production at the level of transcription activation.

Differences in the profile andlevel of transcription factor activity between human and rat osteoblastic cells may account for thelack of an observable transcriptional response to T3 in this heterologous system. We extended our studies of the mechanism by analyzing the actionsand interactions of T3 and IL-1 on the IL-6 promoter. Constructs representing the full-length IL-6 promoter (-779) and shorter sequences (-224. -158, -109) coupled with a luciferase reporterwere transfected into UMR-106 cells using Lipofectamine Plus®. The greatest effects, and an augmented effect in combination with T3 wasfound with the shortest promoter construct tested, which contains the NF-kB response element.In other studies with the -224 promoter, interactions between T3and and another stimulator of resorption, PTH were examined. T3 and PTH effects were additiveor greater. We also showed that estrogen decreased the effects of T3, PTHand T3 +PTH, however in the presence of T3, estrogen had less of an inhibitory effect on theresponse to PTH. Also shown in a study in whichRT-PCR was used to examine effects of IL-1 and T3 on IL-6 mRNA expression in MC3T3-E1cells. There were stimulatory effects with both T3 and IL-1, and there was the suggestion ofpotentiation. We have shown that serum TNF-α levels are elevated in hyperthyroidism independently of the etiology of thyroid hyperfunction

CONCLUSIONS

The maintenance of bone involves a delicate balance between the actions of systemic hormones, local cytokines and growth factors, and physical forces. Disorders that interfere with, amplify, or mimic the effects of these factors can result in abnormal bone remodeling and elicit pathological conditions that result in increased susceptibility to fracture. My research to define the pathways that lead to the responses to these factors, in order to identify new potential targets for therapy.

- Our studies on osteoblasts have focused on parathyroid hormone signaling. PTH can promote both the formation and resorption of bone. Intermittent stimulation by PTH promotes bone formation, likely through the local production of growth factors. Continuous stimulation by PTH promotes resorption through the local production of the osteoclast-stimulatory cytokines RANKL and interleukin-6 (IL-6). PTH acts through specific receptors to activate signaling pathways. Adenylyl cyclase and phospholipase C were previously recognized as signaling molecules activated by PTH-receptor interactions. We have found that the activation of phospholipase D by PTH occurs through the heterotrimeric proteins of the Galpha12 and Galpha13 family and the small G protein RhoA. We have shown that stimulation of the pathway by PTH in osteoblastic cells leads to activation of protein kinase C-alpha and increased IL-6.

- We have found significant differences in the gene expression profiles of the bone tissue of postmenopausal and premenopausal non-osteoporotic women including genes that have not yet been associated with menopausal changes. The separation of the two groups by the multiparametric statistical methods applied suggests the involvement of new candidate gene subsets as well as genetic pathways (canonical TGFB cascade, MAPK and ER signaling) that might be useful for the development of future diagnostic tools. Our findings may provide further insight into the process of postmenopausal changes of bone metabolism as well as it can contribute to the development of new statistical methods for the evaluation of batched genetic data.
- Nuclear triiodothyronine (T3) receptors can be found in osteoblastic cell lines as well as in osteoclastomaderived osteoclasts . Thyroid hormones appear to affect bone either by a direct action on osteoclasts or by acting via osteoblasts . T3 directly stimulates bone resorption in organ cultures that can be inhibited by immunosuppressive compounds , indicating the involvement of immunologically active cytokines. Increased interleukin-6 (IL-6) production, a potent stimulator of osteoclast progenitors, has been reported in these cultures Elevated serum IL-6 and Il-1/ TNF concentrations were seen in hyperthyroid patients as well. A biphasic response in insulin-like growth factor I (IGF-I) production was observed in UMR-106 osteoblastic cells exposed to increasing doses of thyroid hormones: lower concentrations stimulated IGF-I secretion into the medium while higher concentrations inhibited it . Higher than normal serum IGF-I levels have been reported in hyperthyroid patients who exhibited a positive correlation with radius BMD. The anabolic effect of thyroid hormones during growth may be mediated, at least partly, via IGF-I.

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