CLINICAL STUDY

TNF- α gene polymorphism and plasma TNF- α levels are related to lumbar spine bone area in healthy female Caucasian adolescents

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Abstract

Objective: The cytokine tumor necrosis factor alpha (TNF- α) is an important regulator of bone metabolism. Polymorphisms in the promoter region of the TNF- α gene at positions –308 and –863 have been identified. We investigated whether these polymorphisms and circulating TNF- α levels were related to bone mineral density and bone area in adolescent girls.

Design: Bone mineral density (BMD), bone area (BA), anthropometric characteristics and biochemical analyses were measured in adolescent girls and compared with regard to TNF- α genotype.

Methods: Allelic variants of the TNF- α gene in 97 girls, aged 16.9 ± 1.2 years (mean $\pm$ s.d.), were identified using polymerase chain reaction and the restriction endonucleases NcoI and TaiI. Bone mineral density and bone area of the femoral neck, lumbar spine and total body were measured using dual energy X-ray absorptiometry.

Results: Carriers of the rare –863 A allele ($n = 25$) had higher body weight ($P = 0.03$), lumbar spine BMD ($P = 0.02$), and larger total BA ($P = 0.03$), femoral neck area ($P < 0.05$), and lumbar spine area ($P = 0.01$). The independent predictors of BMD and BA were investigated using multiple regression. The TNF- α –863 genotypes ($\beta = 0.18$, $P = 0.03$) and the TNF- α plasma levels ($\beta = 0.19$, $P = 0.04$) independently predicted BA of the lumbar spine but not BA or BMD of any other measured sites. No statistically significant differences in body constitution parameters, biochemical parameters, bone density, or bone area at the measured skeletal sites were found when comparing the groups defined by the allelic variants at position –308 ($P = 0.17$ – 0.84).

Conclusions: We found the TNF- α –863 polymorphism and the TNF- α plasma levels to be independent predictors of lumbar spine area in healthy Caucasian adolescent females.

European Journal of Endocrinology 146 629–634

Introduction

The incidence of osteoporosis is rapidly increasing in most Western societies and the disease affects up to 40% of women and 12% of men at some point during life (1). A common result of the disease is hip fracture, which has doubled in incidence in both sexes since the 1950s (2) and is expected to rise another twofold by the year 2025 (3).

Bone mineral density (BMD) is strongly genetically determined. Twin and family studies have shown that up to 80% of the age-specific variation in BMD is under genetic influence (4, 5). The maximal attained bone mass in life, peak bone mass, accounts for about half of the BMD variation up to 65 years of age (6). Since BMD is a major determinant of future fracture risk (7), finding genetic markers for low peak bone mass in children and adolescents could lead to

improved screening, diagnosis and early preventive measures, such as physical activity regimes and dietary optimization. It is not only mineral density of the skeleton that is important for bone strength. Bone size is a significant determinant for fractures of the vertebral bodies, as Gilsanz *et al.* (8) have shown. Even small changes in size, particularly in external diameter, have a major effect on mechanical properties of a bone (9).

The multifunctional cytokine, tumor necrosis factor- α (TNF- α), has been shown to stimulate bone resorption *in vitro* (10) and in mice (11). The cytokine has been implicated in diseases with increased osteoclastic bone resorption, such as osteoporosis due to loss of gonadal function (12), rheumatoid arthritis (13), periodontitis (14), and loosening of orthopaedic implants (15). Direct evidence of a stimulatory effect by TNF- α on osteoclast formation has been obtained in both

human (16) and mouse (17) bone marrow cultures. In a study by Ota *et al.* (18), linkage was found of a micro-satellite near the TNF- α gene with osteoporosis in 192 Japanese sib-pairs.

Functional polymorphisms at position -308 and -863 in the promoter region of the human TNF- α gene (19) have been reported to be associated with altered TNF- α promoter activity (20, 21) and with different plasma levels of TNF- α in healthy men (21), which may have clinical importance in autoimmune and infectious diseases (22). To our knowledge, these polymorphisms and circulating TNF- α levels have never before been studied in relation to BMD or bone area (BA).

In the present study, we investigated whether these polymorphisms and circulating TNF- α levels were associated with bone density or bone area in healthy Caucasian 17-year-old females, i.e. when all subjects had attained their approximate peak bone mass (23, 24).

Materials and methods

Subjects

From advertisements and information in schools and local sports clubs 97 girls, aged 16.9 ± 1.2 years (mean $\pm$ S.D.), all healthy Caucasians, volunteered to participate in the study. None of the subjects had any disease or medication known to affect bone metabolism. All girls had passed menarche by at least two years. Weight and height were measured using standardized equipment. Using a questionnaire, the average amount of weight bearing physical activity per week was assessed during the last year. Informed written consent was given by all the participants and the study protocol was approved by the Ethical Committee of the Medical Faculty, Umeå University.

Techniques for estimating bone density

BMD (g/cm^2) and BA (cm^2) of the total body, femoral neck, and lumbar spine were measured using a Lunar DPX-L (Lunar Co, Madison, Wisconsin, USA) dual energy X-ray absorptiometer, software version 1.3y. The precision of this method has previously been discussed in detail by others (25). The CV-value (S.D./mean) for repeated measurements is 0.7–2.0% in our laboratory depending on application.

Biochemical analysis

Blood samples were collected after an overnight fast. Osteocalcin was analyzed in plasma samples from 92 girls by a commercially available radioimmunoassay kit (Dia Sorin, Stillwater, MI, USA). All samples were analyzed in duplicate. The sensitivity of this assay was 0.8 ng/ml. Intact parathyroid hormone (PTH)

was measured in all 97 girls, using an Imulite intact PTH, solid-phase sandwich chemiluminescent immunological assay (DPC, CA, USA). Calcium was measured in plasma samples from 94 of the subjects using atomic absorption spectroscopy (26) with 1.2% intra-assay precision. The Double Antibody Estradiol procedure (Diagnostic Products Corp., Los Angeles, CA, USA) was used to determine the levels of estradiol in whole blood. The method uses an anti-estradiol anti-serum raised in rabbit. The radioactive ligand was ^{125}I -labeled estradiol. Two samples from each subject were analyzed and the mean from these samples was then used in further analysis. The samples were analyzed according to the manufacturer's manual, the within-assay coefficient of variation (CV) was 5% and the between-assay CV was 5.5%. The sensitivity of the estradiol assay was 1.4 pg/ml, according to the manufacturer of the assay. Plasma TNF- α levels were measured in 84 girls using the Quantikine HS kit (R&D systems, Minneapolis, MN, USA). The sensitivity of this assay was 0.18 pg/ml, according to the manufacturer.

Genomic DNA analysis

Genomic DNA from the participants was isolated from EDTA stabilized blood using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). Determination of the TNF- α gene polymorphisms at positions -308 and -863 was carried out as previously described (19, 21) with a few modifications. Amplification of the polymorphic sequence was performed with approximately 30 ng genomic DNA, 0.2 mmol/l each of dATP, dCTP, dGTP, dTTP, $1 \times$ PCR buffer and 2.5 U Taq polymerase in a 50 μl reaction mixture (Roche Molecular Biochemicals, Stockholm, Sweden) with 0.4 $\mu\text{mol/l}$ forward primer 5'-AGGCAATAGGTTT-TGAGGGCCAT-3' and 0.4 $\mu\text{mol/l}$ reverse primer 5'-TCCTCCCTGCTCCGATTCCG-3' for the TNF- α polymorphism at -308 and with 0.4 $\mu\text{mol/l}$ forward primer 5'-GGCTCTGAGGAATGGGTAC-3' and 0.4 $\mu\text{mol/l}$ reverse primer 5'-CTACATGGCCCTGTCTTCGT-TACG-3' for the TNF- α polymorphism at -863. For the TNF- α polymorphism at -308, a total of 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C, and 30 s elongation at 72 °C (Peltier Thermocycler, MJ Research, Watertown, MA, USA) were used. For the TNF- α polymorphism at -863, a total of 35 cycles of 30 s denaturation at 94 °C, 1 min annealing at 59 °C, and 1 min elongation at 72 °C were used. The -308G allele creates a cut site for the restriction endonuclease NcoI and the -863A allele creates a cut site for the restriction endonuclease Tail, while the -308A and -863C alleles are left uncut. Ten microliters of the amplified products were cleaved overnight with the restriction endonuclease NcoI (New England Biolabs, Stockholm, Sweden) for TNF- α -308 and the restriction endonuclease Tail (New England

Biolabs) for TNF- α – 863, and electrophoresed and analyzed on ethidium bromide-stained agarose gel.

Statistical analysis

Differences in physical characteristics and bone density between the two groups defined by the TNF- α genotypes were investigated using an independent samples *t*-test, with Bonferroni's correction for multiple comparisons as *post hoc* test if a variable was found significant. Bivariate correlations of the different parameters (such as e.g. weight, height, and physical activity) related to BMD or BA were tested using Pearson's coefficient of correlation. The independent predictors of bone density and bone area were tested using multiple linear regression. The SPSS statistical package for the PC was used in the statistical analyses. A *P*-value less than 0.05 was considered significant.

Results

The TNF- α allelic variants of the polymorphism at position –863 in relation to age, years after menarche, anthropometric characteristics, bone density, bone area and biochemical analysis of the 97 girls are presented in Table 1. There were 72 C/C, 23 C/A and 2 A/A subjects. The allele frequencies were 86.1% for C and 13.9% for A. The genotype distribution was found to be in Hardy Weinberg equilibrium. To further investigate the effects of the A allele, the population was divided into two groups for further analysis: –863C/C

and –863C/A, A/A. Subjects with the A allele were found to have higher body weight ($P = 0.03$) and lumbar spine BMD ($P = 0.02$), and larger total bone area ($P = 0.03$), femoral neck area ($P < 0.05$), and lumbar spine area ($P = 0.01$) compared with subjects without the A allele. No differences in biochemical variables, including TNF- α plasma levels, between the TNF- α – 863 genotypes could be detected.

The independent contributors to the variation in bone density were investigated using multiple regression. Physical activity, weight, height, age, and the two groups of TNF- α allelic variants (–863C/C and –863C/A, A/A) were used as explanatory variables. Age was a stronger predictor (for BA and BMD) than age after menarche and was for that reason used in the multivariate analysis. Physical activity was found to be an independent predictor of BMD at all measured sites ($\beta = 0.33$ – 0.47 , $P < 0.01$). Weight was found to be an independent predictor of total BMD ($\beta = 0.34$, $P < 0.01$) but not of femoral neck BMD or lumbar spine BMD. The TNF- α allelic variants were not independent predictors of BMD at any site ($\beta = -0.10$ – 0.10 , $P = 0.24$ – 0.89).

The independent predictors of bone area were investigated using multiple linear regression. This multivariate analysis included body weight, height, age, physical activity, and TNF- α –863 alleles (Table 2). Body weight predicted bone area of total body ($\beta = 0.54$, $P < 0.01$), and body height predicted bone area of total body ($\beta = 0.43$, $P < 0.01$) and lumbar spine ($\beta = 0.40$, $P < 0.01$). Physical activity predicted bone area of lumbar spine ($\beta = 0.24$,

Table 1 Age, anthropometric data, physical activity, years post menarche, biochemical analysis, bone density and bone area in relation to TNF- α polymorphism at position –863 in 97 seventeen-year-old girls. Differences were investigated using an independent samples *t*-test, with Bonferroni's correction for multiple comparisons. Means and standard deviations are presented.

	Allelic variants, position –863			<i>P</i> -value
	Total <i>n</i> = 97	–863C/A, A/A <i>n</i> = 25	–863C/C <i>n</i> = 72	
Age (years)	16.9 $\pm$ 1.2	17.0 $\pm$ 1.3	16.9 $\pm$ 1.2	0.93
Weight (kg)	59.9 $\pm$ 6.1	62.0 $\pm$ 5.6	59.0 $\pm$ 6.0	0.03
Height (cm)	166.5 $\pm$ 5.1	167.3 $\pm$ 5.3	166.3 $\pm$ 5.1	0.40
Physical activity (hours/week)	4.0 $\pm$ 2.6	4.6 $\pm$ 2.7	3.8 $\pm$ 2.6	0.21
Years after menarche (years)	4.1 $\pm$ 1.4	4.0 $\pm$ 1.6	4.1 $\pm$ 1.4	0.88
Bone density (g/cm ²)				
Total body BMD	1.17 $\pm$ 0.07	1.18 $\pm$ 0.04	1.16 $\pm$ 0.08	0.17
Femoral neck BMD	1.08 $\pm$ 0.13	1.09 $\pm$ 0.11	1.08 $\pm$ 0.14	0.92
Lumbar spine BMD	1.22 $\pm$ 0.12	1.26 $\pm$ 0.09	1.21 $\pm$ 0.12	0.02
Bone area (cm ²)				
Total bone area	2238.5 $\pm$ 166.4	2298.2 $\pm$ 148.8	2217.8 $\pm$ 168.1	0.03
Femoral neck area	5.1 $\pm$ 0.6	5.3 $\pm$ 0.5	5.1 $\pm$ 0.6	<0.05
Lumbar spine area	41.5 $\pm$ 4.2	43.4 $\pm$ 4.2	40.8 $\pm$ 4.0	0.01
Biochemical analysis (plasma)				
Osteocalcin (ng/ml; <i>n</i> = 92)	11.08 $\pm$ 10.15	10.30 $\pm$ 10.98	11.34 $\pm$ 9.93	0.70
Plasma calcium (mmol/l; <i>n</i> = 94)	2.14 $\pm$ 0.08	2.14 $\pm$ 0.06	2.15 $\pm$ 0.08	0.73
Plasma calcium (corrected for albumin; <i>n</i> = 94)	2.15 $\pm$ 0.06	2.15 $\pm$ 0.06	2.15 $\pm$ 0.07	0.93
PTH (pmol/l)	3.59 $\pm$ 1.77	3.38 $\pm$ 1.17	3.66 $\pm$ 1.94	0.40
Estradiol (pg/ml)	55.98 $\pm$ 70.68	50.50 $\pm$ 57.04	57.88 $\pm$ 75.10	0.61
TNF- α (pg/ml; <i>n</i> = 84)	4.95 $\pm$ 1.4	5.06 $\pm$ 1.4	4.91 $\pm$ 1.4	0.67

Table 2 Multivariate analysis showing the independent predictors of bone area at measured sites. Age, weight, height, physical activity, and the different TNF- α allelic variants of the polymorphism at position -863 used as explanatory variables. Linear regression coefficient β - and P -values are presented.

Explanatory variables	Bone area					
	Femoral neck		Lumbar spine		Total body	
	β	P	β	P	β	P
Age (year)	-0.03	0.74	0.20	0.02	0.07	0.17
Weight (kg)	0.09	0.48	0.11	0.29	0.54	<0.01
Height (cm)	0.21	0.08	0.40	<0.01	0.43	<0.01
Physical activity (hours/week)	-0.05	0.61	0.24	<0.01	0.11	0.02
TNF α - 863	0.17	0.10	0.18	0.03	0.04	0.45

$P < 0.01$) and total body ($\beta = 0.11$, $P = 0.02$). The TNF- α - 863 genotypes predicted bone area of the lumbar spine ($\beta = 0.18$, $P = 0.03$) but not of the femoral neck ($\beta = 0.17$, $P = 0.1$) or the total body ($\beta = 0.04$, $P = 0.45$).

The same multivariate analysis was performed including the TNF- α plasma levels, and showed that the TNF- α plasma levels were independently correlated to BA of the lumbar spine ($\beta = 0.19$; $P = 0.04$), but not to BA or BMD of any other site measured. Using linear regression, physical activity ($\beta = 0.38$; $P = 0.001$), but not estradiol levels, was found to predict the TNF- α plasma levels.

No statistically significant differences in body constitution parameters, biochemical parameters, bone density, or bone area at the measured skeletal sites, were found when comparing the groups defined by the allelic variants at position -308 ($P = 0.17$ – 0.84). There were 75 G/G, and 22 G/A subjects. The allele frequencies were 88.7% for G and 11.3% for A. The genotype distribution was found to be in Hardy Weinberg equilibrium.

Discussion

Genetic factors play an important role in the pathogenesis of osteoporosis. Several candidate genes have been studied to examine the effect of allelic variation on bone density. The cytokine TNF- α is an important stimulator of bone resorption (10, 11) by enhancing osteoclast development both through a direct effect on osteoclast precursor differentiation (17) and indirectly by inducing stromal and osteoblastic cell secretion of essential 'downstream' cytokines such as interleukin-6 and interleukin-11 (27). The stimulatory effect by TNF- α on osteoclast differentiation seems to involve both TNF receptor 1 and 2 and, most interestingly, by a pathway independent of RANKL-RANK interaction (17). Recently, a polymorphism in the tumor necrosis factor receptor 2 (TNFR2) was associated with lumbar spine BMD (28). In a study by Ammann *et al.* (29), transgenic mice expressing high blood levels of a soluble

TNF receptor, neutralizing TNF α , was shown to be protected from bone loss caused by estrogen deficiency after ovariectomy. Ammann and co-workers suggested a critical role for TNF- α in the pathogenesis of bone loss induced by estrogen deficiency. Further, estradiol has been shown to directly repress TNF- α transcription (30).

The TNF- α gene is located in the class III region of the major histocompatibility complex (MHC) and has been speculated to contribute to MHC associations with autoimmune and infectious diseases (22). Polymorphisms in the promoter region of the TNF- α gene have been identified (19). In a study by Skoog *et al.* (21), the rare -863 A allele was associated with 31% lower transcriptional activity in a chloramphenicol acetyltransferase (CAT) reporter gene assay, indicating that the -863C/A polymorphism influences the basal rate of transcription of the TNF- α gene. When analyzing serum concentration in 156 middle-aged men, Skoog and co-workers found significantly lower serum TNF- α levels in carriers of the rare -863A allele, indicating an influence on the expression of the gene *in vivo* (21).

In our study, the allelic frequencies of the TNF- α - 863 polymorphism were similar to a previous study (21). Lumbar spine BMD, total bone area, femoral neck area and lumbar spine area were shown to be significantly higher in carriers of the rare -863A allele. A multivariate analysis was performed using age, weight, height, physical activity, and the different TNF- α allelic variants of the polymorphism at position -863 as explanatory variables in which the TNF- α - 863 polymorphism was shown to be an independent predictor of lumbar spine bone area. There was no difference in body height between the allelic variants, indicating an influence of the TNF- α - 863 polymorphism on bone size. Previous studies (8, 31, 32) have emphasized the importance of bone size as a determinant of bone strength. Further supporting the role of TNF- α in determining bone size, is the observed independent correlation of TNF- α plasma levels to lumbar spine area. However, considering TNF- α as a bone resorption stimulator, our finding that high TNF- α plasma levels

are correlated to high lumbar spine area are somewhat contradictory. Our data may suggest that the bone turnover rate and the formation rate are greater in subjects with high TNF- α plasma levels. The correlation between the TNF- α – 863 polymorphism and lumbar spine BMD was lost in the multivariate analysis after being corrected for weight.

In the present study, we could not detect any differences in TNF- α plasma levels between the TNF- α – 863 genotypes. The discrepancies between this result and the findings by Skoog *et al.* (21) could be due to gender differences, since estradiol levels are higher in females than in males and have a direct inhibitory action on TNF- α transcription (30). A limitation of the present study is the relatively small sample size, which may give rise to false associations by chance (type one error), or may fail to detect true differences.

Physical activity was an independent predictor of lumbar spine BA in the present study and has been shown to be correlated to bone area in adolescence (33) and has also been reported to elevate circulating cytokines (34), including TNF- α , following exercise. In the present study, physical activity independently predicted both lumbar spine BA and TNF- α plasma levels, suggesting a possible role of TNF- α in the mechanism by which physical activity may increase bone volume.

In conclusion, this study found the TNF- α – 863 polymorphism and the TNF- α plasma levels to be independent predictors of lumbar spine area in healthy Caucasian adolescent girls.

Acknowledgements

We thank Anette Contardo and Torsten Sandström for excellent technical assistance. This work was supported by the Swedish Center for Sports Medicine Research (CIF), the Swedish Medical Research Council (no. 7225), and the Swedish Rheumatic Association.

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Received 10 September 2001

Accepted 21 December 2001