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Two Promoter Polymorphisms Regulating Interleukin-6 Gene Expression Are Associated with Circulating Levels of C-Reactive Protein and Markers of Bone Resorption in Postmenopausal Women

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IL-6 is a pleiotropic cytokine that plays a critical role in bone resorption. We describe two allelic variants in the IL-6 promoter, −572 and –174 G→C, that alone and in combination influence IL-6 activity *in vitro* and *in vivo*. The association of IL-6 –572 genotypes and –572/−174 G→C haplotypes with serum C-reactive protein (CRP), serum and urinary C-terminal cross-linking of type I collagen (a marker of bone resorption), and osteocalcin (a marker of bone formation) was investigated in a cohort of healthy postmenopausal women (n = 495; mean age ± SD, 72 ± 5.7 yr). Among IL-6 −572 genotypes, CRP was 37% higher ($P = 0.02$) and urinary C-terminal cross-linking of type I collagen was 20% higher ($P = 0.01$) in the presence of the C allele, whereas serum osteocalcin was not different. IL-6 −572/−174 haplotypes (G/C, G/G, and C/G) were significantly associated with all biochemical markers, and additive effects of the two polymorphic loci were found. Thus, there was a significant increase in the level of CRP (up to +79%; $P = 0.007$) and bone resorption markers (up to +32%; $P = 0.017$) with a decreasing number (from four to one) of IL-6 protective alleles –572G and –174C. In addition, there was a trend for lower age-adjusted bone mineral density at the lumbar spine in subjects with less IL-6 protective alleles (up to −9.6%; $P = 0.037$; $P = 0.08$ after further adjustment for weight). In conclusion, we describe two functional polymorphisms in the IL-6 gene regulatory region associated with IL-6 activity in postmenopausal women, both systemically (CRP) and locally in bone. As such, IL-6 polymorphisms are able to influence the risk of osteoporosis as well as other chronic disorders involving IL-6 activity. (*J Clin Endocrinol Metab* 88: 255–259, 2003)

INTERLEUKIN-6 IS A pleiotropic cytokine that plays a central role in immune, inflammatory, and acute phase responses, hemopoiesis, atherogenesis, and several endocrine and metabolic disorders (1). Circulating levels of IL-6 and/or C-reactive protein (CRP), an acute phase response molecule directly stimulated by IL-6, have been associated with the risk of chronic diseases such as osteoporosis (2), type 2 diabetes mellitus (3), and coronary artery disease (4, 5). In bone, IL-6 produced by osteoblasts, monocytes, and T cells promotes osteoclast differentiation and activation (6). IL-6 is tightly regulated at the level of expression by several hormones, cytokines, and their transcription factors. Among them, IL-1 and TNFα activate, whereas estradiol and glucocorticoids repress, IL-6 gene transcription (1). Hence, increased IL-1/TNFα production, such as in rheumatoid arthritis, and decreased estrogen synthesis, as occurs in postmenopausal women, enhance IL-6 expression, bone resorption, and bone loss (6).

Recently, several allelic variants have been identified in the IL-6 gene promoter region (7, 8). Among them, a common G→C polymorphism at position –174 involves a DNA-binding site for NF-IL-6, a transcription factor that can also interact with estradiol/estrogen receptor complexes to regulate IL-6 gene expression. There is some evidence that this polymorphism produces a functional variant, in that the −174 C allele results in lower stimulated IL-6 promoter activity *in vitro* and lower circulating IL-6 concentrations in healthy men compared with the G allele (7). In turn, we have demonstrated that postmenopausal women homozygous for the –174 C allele have low serum levels of C-terminal cross-linking of type I collagen (sCTx), a marker of bone resorption (9).

Due to the evidence that allelic variants forming distinct haplotypes within the IL-6 promoter may cooperate in the regulation of IL-6 transcription *in vitro* (8), we have now investigated another functional G→C polymorphism at position –572 in the IL-6 regulatory region. Although the sequence around –572 does not have a strong homology to any known transcription factor-binding site, there is a potential glucocorticoid receptor element at position –557 to –552 (8). In this study we characterized the association of the IL-6 –572 polymorphism, and particularly of –572/–174 haplotypes, with levels of CRP and markers of bone resorption [sCTx and urinary CTx (uCTx)] and formation (osteocalcin) in an extended cohort of healthy postmenopausal women.

Abbreviations: BMD, Bone mineral density; CRP, C-reactive protein; CTx, C-terminal cross-linking of type I collagen; sCTx, serum C-terminal cross-linking of type I collagen; uCTx, urinary C-terminal cross-linking of type I collagen.

Subjects and Methods

Five hundred seventy-three healthy, community-dwelling women (>94% Caucasians) over the age of 65 yr were recruited from the Greater

Boston area. Entry inclusion and exclusion criteria as well as subjects' clinical characteristics have been described in detail previously (9). Briefly, subjects were excluded if they had received osteoporosis therapy within 1 yr of screening; if they had disorders of the digestive or renal system, cancer, unexplained weight loss, uncontrolled high blood pressure, hyperparathyroidism, malabsorption, abnormal PAP smear, or thromboembolic disease. The study was approved by the ethical committee of the Beth Israel Deaconess Medical Center, and all subjects signed an informed consent.

IL-6 genotypes and haplotypes

DNA was available in 495 women. The –174 G→C polymorphism was determined by PCR amplification and digestion with the restriction enzyme *Nla*III using primers and conditions previously reported (7, 9). The –572 G→C polymorphism was determined by PCR using primers 5′-GGAGACGCCTTGAAGTAACTGC-3′ (forward) and 5′-GAGTTTCCTCTGACTCCATCGCAG-3′ (reverse). The 163-bp fragment was digested with the restriction enzyme *Mbi*I recognizing the –572 G allele to create two DNA fragments of 101/62 bp, which were solved on an 8% Microplate Array Diagonal Gel Electrophoresis (MADGE) gel. Detailed methods for genotyping are available elsewhere (10).

Haplotypes were inferred using the Haplotyper V2 program, which uses a Bayesian algorithm to reconstruct phases, as recently described (11).

In vitro IL-6 gene expression/reporter gene constructs

To determine the influence of the –572 G→C allelic variants on IL-6 gene expression *in vitro*, a 1.2-kb fragment of the IL-6 5′-untranslated region (−1182 to +13) containing the –572G/−174G allelic variant (12) was subcloned in the *Sac*I/*Xho* restriction sites of Basic luciferase reporter (Promega Corp., Madison, WI). The –572C allele was then created by site-directed mutagenesis using QuickChange (Stratagene, La Jolla, CA) and primers 5′-GTTCTACAACAGCCCCTCACAGGGAGAGCC-3′ (forward) and 5′-GGCTCTCCCTGTGAGGGGCTGTTGTAGAAC-3′ (reverse). The orientation and integrity of the IL-6 promoter-luciferase reporter construct was verified by DNA sequencing.

HepG2 cells in 96-well plates were transfected with 0.1 μg DNA of each IL-6-reporter construct and 0.001 μg DNA of pRL-TK Renilla luciferase expression plasmid, a control for transfection efficiency, using lipofectin (Life Technologies, Inc., Paisley, UK). After 24 h, cells were treated with IL-1β (100 U/ml) and dexamethasone (0.5 μM) for 3–24 h to stimulate IL-6 gene expression, after which cells were harvested and assayed for luciferase activity on a Tropix Microplate Luminometer (Perkin-Elmer Corp., Norwalk, CT). Values were adjusted for background fluorescence and transfection efficiency.

Biochemical markers

Fasting serum and urine samples were available for biochemical determinations in 485 and 423 subjects, respectively. Serum CRP was measured using an ultrasensitive immunonephelometry method (N Latex CRP mono, Behringwerke AG, Marburg, Germany) on a BNA Behring nephelometer. The intra- and interassay variations were less than 5%, and the detection limit was 0.2 mg/liter. Four subjects (0.8% of the study population) whose CRP values were greater than +4 SD from the population mean (*i.e.* >32 mg/dl, up to 113 mg/dl) were considered to have a potentially undetected inflammatory disorder and subsequently discarded from analysis.

CTx was measured in serum by a two-site assay using monoclonal antibodies (Elecsys, Roche, Mannheim, Germany), and uCTx was determined by a competitive immunoassay on an automatic analyzer (Enzymum test, β Crosslaps, Roche); values were corrected for creatinine levels. Serum osteocalcin was measured by a two-site assay, measuring both intact osteocalcin and its main proteolytic fragment (Kryptor-Osteo, CIS-Bio International, Gif/Yvette, France). Intra- and interassay coefficients of variation for these assays have been previously reported (9).

Bone mineral density (BMD)

Baseline BMD (grams per square centimeter), measured by dual energy x-ray absorptiometry (DXA, QDR-4500A, Hologic, Inc., Waltham, MA), was available for 417 patients with complete genotypes. BMD values for the total hip and lumbar spine were considered for analysis in the present study.

Statistical analysis

Due to the low prevalence of the –572 C allele in our population, no CC homozygotes were detected. Hence, differences in biochemical measurements between IL-6 –572 genotypes GG and GC were analyzed by two-tailed *t* test for unpaired comparisons, using the Bonferroni correction for multiple comparisons. In this case, $P < 0.015$ was considered statistically significant. Association of IL-6 –572/−174 haplotypes with biochemical measurements was analyzed by multiple regression analysis including haplotypes transformed into dummy variables (G/C, 1; G/G, 2; C/G, 3), age, and CRP (when appropriate) as the independent variables. Furthermore, an allelic score was determined by counting the number of protective alleles (*underlined*), *i.e.* the number of –572G;174C alleles in each subject, giving a score of 1–4 as follows: a1 = GC;GG, a2 = GG;GG or GC;GC, a3 = GG;GC, and a4 = GG; CC. Differences in baseline characteristics, BMD, and biochemical measurements were analyzed by analysis of covariance using allelic scores as categorical groups and adjusting for age and CRP (the latter when appropriate) and also by regression analyses, adjusting for age and weight in which a linear additive effect of the number of protective alleles was assumed, *i.e.* that the effect of a2 was twice that of a1, the effect of a3 3 times that of a1, and the effect of a4 twice that of a2 (and 4 times that of a1).

Results

IL-6 −572 G→C alleles influence gene expression levels in vitro

To evaluate the functional significance of the −572 G→C alleles on IL-6 gene expression levels *in vitro*, IL-6 promoter-luciferase reporter constructs were transiently expressed in HepG2 cells (Fig. 1). Unstimulated IL-6 promoter activity was similar in cells expressing the –572G and C variants. In contrast, IL-1β stimulation in the presence dexamethasone induced 3- and 2-fold increases in IL-6 promoter activity after 8 h in cells expressing the –572C and –572G alleles, respectively, this difference being highly significant ($P = 0.0001$ overall; $P = 0.006$ at 8 h). A similar difference in transcriptional activity in the two constructs was observed after stimulation with TNFα and dexamethasone (not shown).

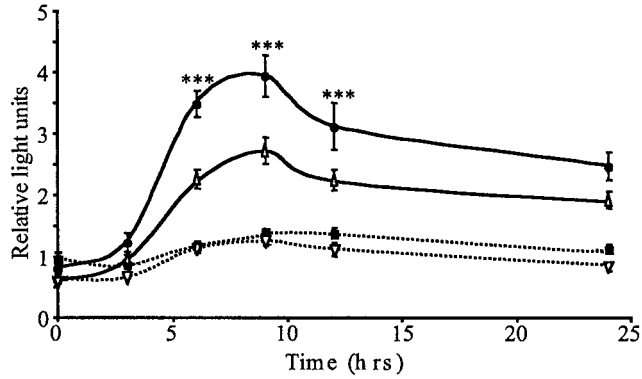

FIG. 1. Influence of IL-6 promoter polymorphisms on gene expression levels *in vitro*. Promoter-luciferase reporter constructs containing the IL-6 −572C (*closed symbols*) or G (*open symbols*) allele were transiently expressed in HepG2 cells. Luciferase activity was measured at baseline and at the times indicated for up to 24 h in the absence of stimulation (*dotted lines*) or after promoter activation by IL-1β (100 U/ml) and dexamethasone (500 nM; *solid lines*). Results are expressed as mean relative light units ± SEM from eight separate experiments and are corrected for transfection efficiency as indicated in *Materials and Methods*. ***, $P = 0.0001$ for the overall difference between –572C and G alleles at 5, 8, and 12 h after activation.

Association of IL-6 –572 genotypes with CRP and markers of bone turnover

Among 573 healthy, late postmenopausal women, DNA was available in 495 (mean age ± SD, 71.9 ± 5.7 yr; range, 64.6–94.8 yr). Among these, 456 could be unambiguously genotyped for the –572 G→C polymorphism. The prevalence of G and C alleles was 94% and 6%, respectively. Accordingly, no CC homozygote was detected in our population. Age was similar in GG (n = 400) and GC (n = 56) subjects (71.8 ± 5.8 and 72.9 ± 5.8 yr). CRP, osteocalcin, sCTx, and uCTx levels all tended to be higher in GC compared with GG subjects (Table 1). After Bonferroni's correction for multiple comparisons, differences were statistically significant for uCTx (+20% in GC; $P = 0.01$) and were of borderline significance for serum CRP (+37% in GC; $P = 0.02$).

Correlation of IL-6 –572/−174 haplotypes with biochemical measurements

The prevalence of IL-6 –174 G and C alleles was, respectively, 61% and 39%. CRP levels were nonsignificantly increased (+27%; $P = 0.27$) in genotypes –174 GG (n = 180) and GC (n = 208) compared with homozygotes CC (n = 79). Serum CTx levels were significantly higher in GG compared with CC (+24%; $P = 0.016$), whereas osteocalcin did not differ among IL-6 –174 genotypes, in keeping with our previous observations in a smaller group of subjects from this cohort (9).

The –572C allele was observed to always occur on the –174G allele, in keeping with previously published data (8, 10). Three common −572/−174 haplotypes were therefore estimated by computer program (11) to be present in our population, namely G/C, G/G, and C/G (n = 337, 516, and 55, respectively). Multiple regression analysis indicated that IL-6 haplotypes were positively correlated with all biochemical measurements [regression coefficients ± SEM: CRP, 0.526 ± 0.222 ($P = 0.018$); sCTx, 0.027 ± 0.012 ($P = 0.02$); uCTx, 0.696 ± 0.298 ($P = 0.02$); osteocalcin, 1.219 ± 0.564 ($P = 0.03$)]. Age was also significantly correlated with all biochemical measures (data not shown), whereas CRP was negatively correlated with uCTx (−0.145 ± 0.046; $P = 0.002$) and OC (−0.357 ± 0.086; $P = 0.0001$), but was not correlated with sCTx.

TABLE 1. IL-6-572 G>C genotypes and levels of CRP and bone markers in postmenopausal women

	GG	GC	*P*
CRP (mg/liter)	3.28 (0.18)	4.52 (0.76)	0.023
uCTx (nM/mM cr)	8.88 (0.26)	10.65 (0.69)	0.010
SCTx (ng/ml)	0.333 (0.010)	0.355 (0.027)	NS
Osteocalcin (ng/ml)	21.26 (0.50)	23.20 (1.39)	NS

Means (SEM). NS, Not significant; cr, creatinine.

Additive effects of IL-6 –174 and –572 alleles on biochemical markers and BMD

Subjects were further segregated according to an allelic score, taking into account the number (from 1–4) of protective alleles –572G and −174C. Baseline clinical characteristics were similar among allelic scores (Table 2). In contrast, there was a trend for age-adjusted lumbar spine BMD to be decreased in subjects with a lower allelic score (9.6% lower in carriers of one compared with four protective alleles; $P = 0.037$; $P = 0.08$ after further adjustment for weight), whereas total hip BMD showed a similar, but nonsignificant, trend (Table 2). Additive effects of IL-6 –572 and –174 polymorphisms were mostly noted on CRP and bone resorption markers (Fig. 2). Thus, compared with subjects with all four protective alleles, subjects with an allelic score of 1 had age-adjusted CRP, uCTx, and sCTx values that were 79% ($P = 0.007$), 32% ($P = 0.017$), and 30% ($P = 0.05$) higher, respectively.

Discussion

These results taken together with our previous observation that IL-6 –174 genotypes are associated with levels of bone resorption (9) indicate that functional G→C gene polymorphisms at positions –572 and –174 in the IL-6 promoter region influence IL-6 activity in postmenopausal women. In this study we observed parallel responses in IL-6 gene expression levels related to –572 G and C alleles *in vitro* and levels of CRP and bone resorption markers *in vivo*. Indeed, the –572 C allele, which showed an increased promoter activity in response to IL-1β (and also TNFα) in transfected cells, was associated with higher levels of CRP and uCTx in these women. These observations are compatible with higher IL-6 activity in carriers of the –572C allele, both systemically to induce hepatic production of CRP and locally to activate osteoclastic bone resorption (6). More importantly, allelic polymorphisms in the IL-6 promoter region were found to

TABLE 2. Baseline clinical characteristics and BMD according to IL-6 allelic scores

	A1	A2	A3	A4
No.	36	163	186	66
Age (yr)	72.7 (5.5)	71.5 (6.1)	72.4 (6.1)	71.3 (4.5)
Age range	(65.1–89.9)	(64.9–90.8)	(64.6–94.8)	(64.9–81.7)
Weight (kg)	70.0 (11.7)	68.4 (14.9)	70.0 (17.8)	70.2 (13.9)
Height (cm)	158.2 (6.4)	159.0 (6.7)	158.7 (6.2)	159.6 (6.2)
BMI (kg/m^2)	28.0 (4.5)	27.0 (5.0)	27.8 (6.8)	27.5 (4.9)
Calcium intake (mg/d)	957.5 (423.6)	846.6 (389.8)	921.3 (463.4)	845.7 (414.2)
Vitamin D intake (IU/d)	299.3 (216.8)	237.9 (165.5)	263.6 (209.8)	269.8 (176.2)
Spine BMD (g/cm^2)	0.856 (0.130)	0.898 (0.186)	0.899 (0.162)	0.946 (0.173)[a]
Hip BMD (g/cm^2)	0.775 (0.136)	0.797 (0.148)	0.794 (0.135)	0.826 (0.136)

Means (SD). Allelic scores (A1 to A4) refer to the number of IL-6-174C and -572G protective alleles (see *Subjects and Methods* for details). BMI, Body mass index.

[a] $P = 0.037$ and $P = 0.08$ for, respectively, age-adjusted and age- and weight-adjusted BMD differences among allelic scores.

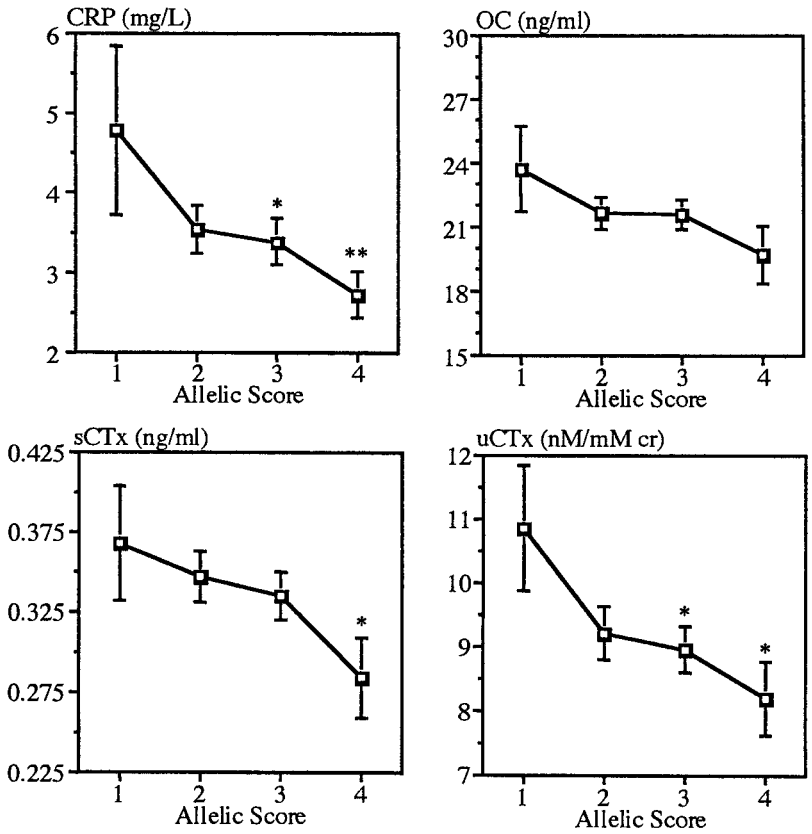

FIG. 2. Additive effects of IL-6 allelic polymorphisms on levels of CRP and bone markers. Results are the mean (±SEM) of biochemical measurements associated with allelic scores reflecting the number of IL-6 protective polymorphisms –572G and –174C (1, n = 35; 2, n = 155; 3, n = 175; 4, n = 63). *, $P \leq 0.05$; **, $P \leq 0.01$ (for age-adjusted values compared with allelic score of 1, by ANOVA).

cooperate in the regulation of IL-6 activity *in vivo*, in keeping with the recent observation that IL-6 promoter haplotypes (rather than simply single variant sites) influence IL-6 gene expression *in vitro* (8). This was shown by the significant association of IL-6 –572/−174 haplotypes with biochemical markers as well as by additive effects of protective –572G and −174C alleles on decreasing levels of both CRP and bone resorption markers and also possibly on BMD. Moreover, the lack of correlation between CRP and sCTx levels suggests that association of IL-6 polymorphisms with both CRP and bone resorption markers is largely independent and may reflect distinct regulation of IL-6 gene expression in the liver and skeleton. Thus, the observation that IL-6 polymorphisms were not so strongly associated with the level of osteocalcin, a marker of osteoblastic activity, may be explained by the fact that IL-6 primarily activates osteoclastic bone resorption and that bone resorption and formation are poorly coupled in late postmenopausal women (6). In this context, the lower lumbar spine BMD in carriers of IL-6 promoter variants −572C and –174G might reflect an accelerated bone turnover affecting primarily the trabecular bone compartment.

Numerous candidate genes have been investigated in relation to BMD and osteoporosis (see Ref. 13 for review). Among them, very few polymorphisms were shown to be functional allelic variants (as opposed to silent allelic markers), and virtually none has been consistently associated with the rate of bone turnover. Yet, bone resorption, particularly as it accelerates after the menopause, is a major determinant of the osteoporosis risk (14). In bone, IL-6 produced by osteoblasts and mononuclear cells is a critical factor to promote osteoclast differentiation and activation (6). Hence, after the menopause, increased IL-6 production is associated with increased bone resorption and bone loss (2). In keeping with these observations, a few studies have identified the IL-6 gene locus to be linked with bone mass in postmenopausal women (15, 16), but not in younger females (17). Our own results now provide the basis for a genetically determined mechanism, the regulation of IL-6 gene expression by its promoter polymorphisms, to support these observations.

In conclusion, this study suggests that IL-6 gene promoter polymorphisms –572 and –174 G→C influence levels of circulating CRP and bone resorption markers. As such, these variants are potential genetic susceptibility factors that need to be further evaluated with regard to the risk of osteoporosis in the elderly and of other chronic diseases in which IL-6 activity has been clearly implicated, including cardiovascular diseases (18) and diabetes (3).

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