

The influence of IL-6 and TNF- α genes promoter region polymorphisms (G174C – IL-6 and G308A – TNF- α) on the level of cytokines in obese and normal weight subjects

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Abstract

Proinflammatory cytokines level, including IL-6 and TNF- α , is changing significantly during hyperplasia and hypertrophy of adipocytes. The increase in IL-6 and TNF- α concentration is a direct result of increased expression of their genes. Gene polymorphisms, especially those localized in transcription regulating regions such as promoters, are among factors influencing gene expression profile. The aim of the study is to assess the influence of polymorphic changes in promoter regions: G174C in IL-6 gene and G308A in TNF- α gene on the level of the cytokines in obese and normal weight subjects.

The study included 102 obese subjects and 77 normal weight subjects. Genotyping was performed using PCR-RFLP technique, while cytokine level was assessed using ELISA assay. The obtained results showed the connection between a certain genotype and IL-6 and TNF- α genes expression level. In non obese subjects with C/C genotype IL-6 level was lower than in subjects with G/C genotypes. In contrary in TNF- α gene the difference was found in the group of obese. The A allele carriers were characterized by higher TNF concentration. However, many additional modifying factors, which often conceal the influence of polymorphisms on gene expression, should be considered.

Key words: obesity, interleukin-1, interleukin-6, tumor necrosis factor α , gene polymorphism.

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Introduction

Interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α) are proinflammatory cytokines influencing various processes in the organism. Both cytokines influence immune response and are responsible for development and maintenance of inflammatory reactions.

Interleukin-6 takes part in stimulation of acute phase proteins synthesis in liver, B cell differentiation into antibody-secreting cells, activation of T cells recognizing antigen and influencing T cell differentiation into cytotoxic T cells. Whereas TNF works mainly as a cytotoxic/cyto-

static agents for malignant cells and activates inflammatory response [1, 2]. In cooperation with other cytokines e.g. IL-1 or IL-6, TNF- α increases proliferation and differentiation of T and B cells. It also stimulates proliferation and cytotoxicity of NK cells and cytotoxic T cells.

Biological activity of both factors depends on their concentration, which is a direct result of gene expression level.

In human genome, IL-6 gene maps to 7p21-p14, between D7S135 and D7S370 markers [3]. It is approximately 5 kB in length and consists of 5 exons [4]. Interleukin-6 promoter structure contains many regulatory sites (response elements) which allows induction of gene expres-

sion via many regulatory factors e.g. glucocorticosteroids or cAMP [5, 6]. Extremely important response element is NF- κ B binding site, which is responsible for induction of IL-6 expression by IL-1 and TNF, especially in non-lymphoid tissue [7, 8]. Both in encoding sequence as well as in regulatory sites numerous single nucleotide polymorphisms (SNPs) were observed. One of the most commonly found and analyzed SNPs is 174 G/C in the promoter region.

Gene encoding TNF maps to human chromosome 6 (6p21.3) in close proximity of genes encoding MHC molecules. Some authors suggest, that TNF is encoded within MHC gene complex, between MHC class I and MHC class III gene complexes. Human TNF gene consists of 4 exons and 3 introns and is 2,762 base pairs long. mRNA for TNF is 1,669 base pairs long. 3'-UTR (untranscribed region) in mRNA contains repeats of "AUUUA" motif, which is a common element present in short half-life transcripts. In the promoter region many NF- κ B binding sites were recognized as well as enhancers and silencers of transcription [9-11].

In encoding sequence as well as in promoter and regulatory regions many SNPs were recognized. The presence of certain polymorphisms was connected with the presence or predisposition towards some diseases such as generalized infections, malaria, obesity and especially type 2 diabetes and insulin resistance. However, SNPs localized in promoter region was connected mainly with the influence on the TNF expression level.

The increase in proinflammatory cytokines level is connected with higher risk of insulin resistance and type 2 diabetes. TNF regulates influence of insulin on adipose tissue and G308A polymorphism may lead to change in fat storage pattern in adipocytes. The increased expression of proinflammatory cytokines is observed in overweight and obese subjects. Simultaneously, some authors indicate the differences in tested polymorphisms frequency in obese group in comparison to controls (normal weight). In this context, it is extremely interesting to assess the influence

of promoter region polymorphisms among IL-6 and TNF- α genes on expression level of this cytokines.

Material and methods

The study included 102 obese subject, body mass index (BMI) > 25 : 74 males aged from 31 to 77 years (mean 52.5 years; SE \pm 1.06, SD \pm 9,1) and 28 females aged from 39 to 75 years (mean 60.6 years; SE \pm 1.5, SD \pm 8.4).

Control group included 77 healthy subjects with normal weight, BMI < 25: 39 males aged from 22 to 72 years (mean 39.3 years; SE \pm 2.3, SD \pm 14.8) and 38 females aged from 23 to 78 years (mean 46.6 years SE \pm 2.4, SD \pm 15.3).

In the studied population no allergic, hematologic or other immunologic diseases (including malignancies) were found. The tests were performed in the state of good health, with no signs of infection and at least 3 weeks after last infection, antibiotic treatment or vaccination. During the study patients did not take any antioxidants nor immunomodulating agents and presented with normal blood count results.

Genetic tests

Genomic DNA isolation was performed using Genomic Midi AX, A&A BIOTECHNOLOGY (Gdynia, Poland).

DNA fragments amplification was performed using PCR method with appropriate starters. Polymorphisms were identified using PCR-RFLP technique. PCR products were digested with appropriate restriction enzymes: for IL-6 G174C – LweI and for TNF- α G308A – NcoI (Tables 1, 2).

Determination of interleukin-6 and tumor necrosis factor concentration

Blood samples were collected using EDTA as anticoagulant. To determine blood plasma concentration of IL-6 and TNF respectively "Quantikine human IL-6" and "Quantikine human TNF" R&D Systems (MN, USA) ELISA assay kits were used. The procedure was performed according to manufacturer instruction.

The optical density was estimated using Microplate Reader Stat Fax 2100, Awareness Technology Inc. (USA).

Statistical analysis

The obtained results were analyzed in the whole population as well as in relation to gender and compared to control group.

The frequency of each genotype was assessed using chi-square statistical analysis. The influence of genotypes on anthropomorphic and biochemical results were analyzed

Table 1. PCR conditions for each gene

PCR stage	IL-6	TNF
Initial denaturation	94°C	94°C
	10 min	3 min
Denaturation	94°C	94°C
	1 min	1 min
Annealing	55°C	60°C
	35 s	1 min
Elongation	72°C	72°C
	1 min	1 min
Cycles number	35	36
Final elongation	72°C	72°C
	10 min	5 min

Table 2. Restriction enzymes digestion conditions

Enzyme	Units number (U)	Time (hours)	Temperature
LweI	1	24	37°C
NcoI	2	24	37°C

using nonparametric ANOVA analysis – Kruskal-Wallis test and Mann-Whitney test. The results were considered as statistically significant when p -value was $\geq 0,05$. The calculations were performed using STATISTICA software and Microsoft Excel.

Results

G174C polymorphism analysis in promoter region of IL-6 gene showed that C allele was more frequent in obese subjects. In the whole group of obese subjects the frequency of C allele analyzed in homozygotes C/C and heterozygotes G/C was higher than in control group, however the difference did not reach statistical significance ($p = 0,07$).

The significant difference in the frequency of genotypes with C allele (both homozygotes C/C as well as in heterozygotes and homozygotes together (C/C + G/C) was found in men (Figure 1).

Opposite, the analysis of obese women group showed no significant differences in the frequency of each allele.

Mean IL-6 level in control group was lower in comparison to obese group. However, the statistical significance was found only between obese and normal weight women ($p = 0,001$) (Figure 2).

The influence of IL-6 gene polymorphism on IL-6 synthesis was assessed in carriers of each genotype: G/G, G/C and C/C. In addition, the influence of G and C allele in homo- or heterozygotes on IL-6 concentration was assessed. In the control group we found some differences in IL-6 level depending on the genotype. In subjects with C/C genotype IL-6 level was lower than in subjects with G/C genotypes. The differences in the whole population were statistically significant ($p = 0,05$) (Figure 3). The similar relationship was found in the group of males, however it was not confirmed in the analyzed group of women.

The analysis performed in obese subjects group showed no statistically significant difference in IL-6 level depending on genotype.

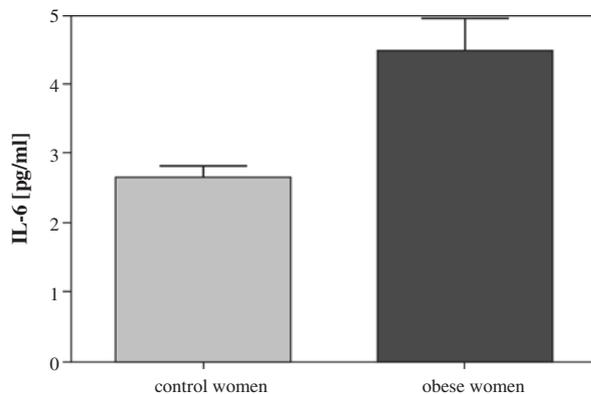


Fig. 2. IL-6 level difference in obese and normal weight women ($p = 0,001$)

In the analyzed population no significant difference in the frequency of G/G and G/A genotypes in promoter region of TNF- α gene between obese subjects and control group was found. The obtained results showed a slightly higher frequency of the allele A in obese subjects in comparison to controls. The difference varied from 5 to 7%.

The frequency of allele A in the whole analyzed population of obese subjects is 5% higher than in normal weight group. In obese females the frequency of allele A is approx. 6% higher than in controls. In obese males, similarly to obese females group, the G/A genotype was more frequent (approx. 6%). None of these differences was statistically significant.

The TNF secretion level in obese subjects was higher than in control group. All obtained results were of highly statistically significant (Figure 4). The highest difference, more than four-times, was found in the group of women.

The comparison of mean TNF concentration between allele A carriers and G/G homozygotes from the control

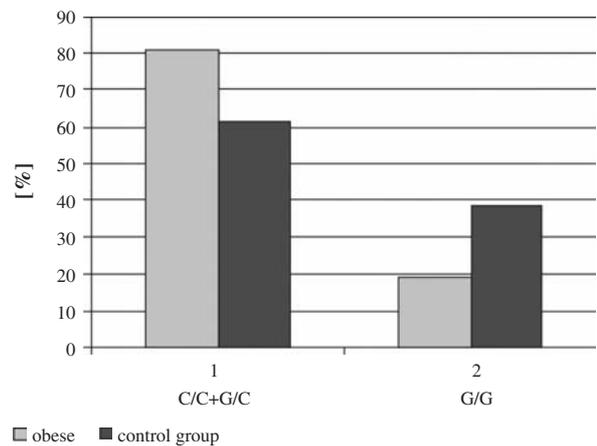


Fig. 1. Percentage participation of C allele in homo- and heterozygotes in males from obese and control group ($p = 0,02$)

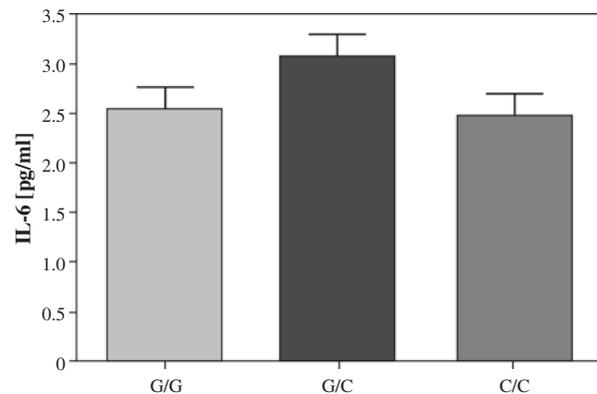


Fig. 3. The relationship between IL-6 level and genotype of IL-6 gene in control group (normal weight subjects) ($p = 0,05$)

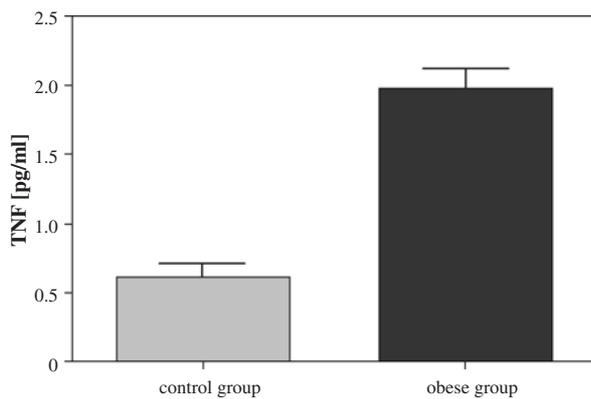


Fig. 4. The mean TNF level in control group and obese subjects group ($p = 0,0005$)

group showed no significant differences. On the other hand, in obese population, a significant difference in TNF concentration between allele A carriers and G/G homozygotes was found. In all analyzed groups allele A carriers were characterized by higher TNF concentration. The difference was statistically significant in obese subjects population ($p = 0,03$) (Figure 5).

Discussion

In the present paper we have found the differences in IL-6 concentration depending on the polymorphic changes in IL-6 gene. The lowest concentration of IL-6 were observed in C/C homozygotes, slightly higher concentration in G/C heterozygotes and the highest in G/G homozygotes. However, the described relation was found only in the control group. Interestingly, IL-6 concentration was higher in obese subjects in comparison to controls. Presumably, in obese subjects several factors secreted by adipose tissue, such as TNF, can influence the IL-6 level. The increase in secretion of such adipokines can stronger modify gene transcription level than the polymorphism examined in our study. Our results are consistent with those published by Fishman, who showed the influence of G174C polymorphism on the IL-6 expression level [12]. He found, that non-stimulated cells obtained from C allele homozygotes showed significantly lower gene expression than cells from G allele carriers. After IL-1 or lipopolysaccharide stimulation, IL-6 expression in allele G carriers was 2-3 times increased in comparison to the level before stimulation. In C allele carriers the stimulation did not cause significant increase in IL-6 expression. Moreover, Fishman found that in G/G homozygotes IL-6 level in bloodstream is almost 2-times higher than in C/C homozygotes. This phenomenon can be caused by the fact, that the region localized between -225 and -164 points,

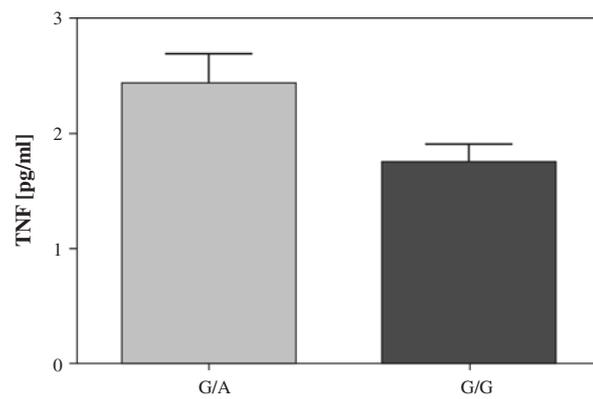


Fig. 5. The mean TNF concentration in allele A carriers as well as in G/G homozygotes for TNF gene in obese subjects group ($p = 0,03$)

which contains the polymorphism G→C, negatively affects gene expression regulation [13].

The research on inhibition of IL-6 gene expression showed, that glucocorticosteroid receptor binds to the region localized in -201 area. G174C polymorphism appears to be localized close enough to inhibit transcriptional activity via glucocorticosteroid receptor binding site. Moreover, replacement of G for C in -174 position can create a new binding site for NF-1 transcription factor. NF-1 is able to modify transcription process. In case of IL-6 gene it seems to be inhibitory effect. Interleukin-6 plasma concentration depends on gene expression changes [14]. Interleukin-6 transcription level is precisely regulated by several factors such as: NFIL-6, NFκB, Fos/Jun, CRBP and glucocorticosteroid receptor. The experiments performed on cell lines showed that the region from -180 to -123 in the gene promoter, plays a key role in virus-, cytokines- (IL-1 and TNF), platelet growth factor- (PGF)- and epidermal growth factor- (EGF)-induced transcription [15, 16]. The activation of IL-6 gene promoter is based on the cooperation between NFIL-6 transcription factor (-158 to -145) and NF-κB transcription factor (-73 to -63) [17, 18]. The inhibition of IL-6 expression by steroid hormones, such as glucocorticosteroids or estrogens, is presumably connected with interaction between estrogen or glucocorticosteroid receptor complex and NFIL-6 and NF-κB transcription factors [13, 19]. In consequence, it can lead to blockade of their binding to DNA and inhibition of transcription [20]. The existence of polymorphic sites in gene promoter can lead to disturbances in gene expression regulation and in consequence result in differences in protein secretion in subjects with various genotypes. The impact of steroid hormones on IL-6 expression can explain different influence of G174C genotype on cytokine expression level in men and women. In our study the highest influence of C/C genotype on obesity was found in the

group of adult men. In the group of adult women the connection was not distinct. The result suggests that sexual hormones exerts higher influence on IL-6 expression than G174C genotype. The study performed by Terry *et al.* [21] showed, that IL-6 gene transcription regulation is characterized by high tissue specificity. It explains the significant differences in correlations between genotypes and each disease entity. The described study suggest, that G174C polymorphism should be analyzed in parallel to other polymorphic sites localized in IL-6 promoter. It was found, that stimulation of C/C genotype cells with IL-1 causes increase in gene expression. This observation is inconsistent with Fishman *et al.* [12]. Lower or higher IL-6 gene expression is connected with the presence of other polymorphisms, such as AG8/12G and GG9/11G, which exerts significantly higher influence on gene expression. In this context, the assessment of the most frequent haplotypes and their influence on cytokine expression level seems to be essential. We suspect, that –174 G allele alone is not responsible for increase in gene expression, but it exerts synergic effect with other polymorphic gene variants e.g. AnTn –392 to –373 [21]. The described influence can vary between different types of cells. Taking the above into consideration we conclude, that the influence of G174C polymorphism on IL-6 expression level in different cell types can depend on additional factors such as acute inflammation or obesity. This hypothesis is confirmed by the results of other studies showing the increase in IL-6 expression in C/C carriers after by-pass grafting. However, the described increase was not observed before grafting procedure.

Interleukin-6 expression is regulated by the several factors. Interleukin-1 and TNF cause increase in IL-6 expression, what is the reason of high IL-6 levels during chronic inflammatory diseases such as rheumatoid arthritis. On the other hand, hormones such as estradiol or glucocorticosteroids inhibit IL-6 gene transcription [19], what can be observed in postmenopausal women and can be the trigger for osteoporosis.

The functional role of G308A polymorphism is not fully elucidated. It is localized inside the sequence that is bound by AP-2 transcription factor in TNF gene promoter [22]. Many authors connect this localization with enhancement of basal and inducible TNF gene transcription in adipose tissue [23]. Because transcription rate is a key factor influencing TNF expression, the presence of polymorphic site in the gene regulatory region can significantly contribute to differences in its expression. Our results also indicate that there is a connection between presence of allele A in polymorphic site –308 and increase in TNF expression. In obese subjects group allele A carriers had significantly higher TNF plasma concentration ($p = 0,008$) in comparison to G/G homozygotes. It is worth noticing, that in case of obese subjects, both humans and animals [24] level of TNF secretion increases and the increase depends on percentage content of adi-

pose tissue in the body [25]. This relation was observed in our study, where TNF secretion level in obese and overweight subject was significantly higher in comparison to control group.

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