English version: ANALYSIS OF SPECIFIC GROUPS OF MICROORGANISMS ISOLATED FROM ATHEROSCLEROTIC CORONARY ARTERIES OF PATIENTS DEPENDING ON ASP299GLY POLYMORPHISM OF TLR4 GENE*

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The aim of this work was to establish the presence of specific groups of microorganisms, including paradontopathogenic in atherosclerotic plaque and surrounding tissues and the dependence of microbial contamination of polymorphism 896A / G gene TLR4 (rs4986790), and to identify the possible relationship of atherosclerosis and Asp299Gly gene polymorphism TLR4 in patients with coronary heart disease (CHD). Materials and methods: The 31 autopsies of coronary arteries from patients whodied from coronary arteries disease and 5 from healthy individuals. Results and discussions: The presence of periodontal pathogenic microorganisms in atherosclerotic plaques was observed in 83,9%. In 51,6% two or more species of microorganisms were found. Only in 11,1% samples with atherosclerotic plaques the microorganisms were found in undamaged tissues. The patients who died from coronary arteries disease had 299Gly allele of TLR4 gene more frequent than healthy individuals (p=0,04) OR 2,92 (1,15-7,41). The presence of TLR4 gene polymorphic allele G in the individual genotype determines the increased contamination of atherosclerotic plaque tissues by the representatives of the following genera: Lactobacillus sp., Enterobacterium sp., Sneathia sp. /Leptotrihia sp. /Fusobacterium sp., Mobiluncus sp. /Corynebacterium sp., Peptostreptococcus sp. The emergence of new correlation pairs with participation of Lachnobacterium sp. /Corynebacterium sp. among the carriers of G allele has been revealed via the intragroup correlation analysis. Conclusion: The obtained results confirm the possible involvement of the represented groups of microorganisms in the pathogenesis of atherosclerosis and the role of the TLR4 gene polymorphic variant G in the increased microbial contamination of the coronary arteries tissues. The presence of 299Gly allele of TLR4 gene increases the risk of this disease.

Key words: periodontal pathogenic microorganisms, polymorphism, Toll-like receptor 4, atherosclerosis.

Recent studies have convincingly demonstrated that pathogenesis of atherosclerosis is based on a chronic inflammation that causes endothelial damage and formation of atherosclerotic plaques in the artery wall. Therefore, increased attention is paid to infectious agents as possible etiological factors of initiation and progression of atherogenesis [3 , 7].

The concept of atherogenesis initiation through an interaction of endogenous and exogenous microbial ligands with Toll-like receptors (TLRs) gains an ever increasing importance. A genetic variability of TLR can determine differences in the susceptibility of organisms to bacteria and viruses as well as in the intensity of the inflammatory process [9].

The aim of the present study was to establish the presence of specific groups of microorganisms, including periodontal pathogens in the atherosclerotic plaque and surrounding tissues, investigate a dependence of microbial contamination on 896A / G gene TLR4 (rs4986790) polymorphism as well as to identify possible relationship of atherosclerosis and Asp299Gly polymorphism of the TLR4 gene in patients with ischemic heart disease (IHD).

Materials and methods

31 aseptically obtained autopsy tissue samples of coronary arteries from people who died of IHD were investigated. The controls were people who died of causes unrelated to IHD. The study was conducted with the permission of the Commission on Bioethics of State Higher Educational Institution of Ukraine "Ukrainian Medical Stomatological Academy".

DNA isolation from tissue lysates was performed with a DNA extraction kit for the biopsies "Helikopol" ("Litech", Moscow). The obtained DNA solutions were used for further qualitative detection of periodontal pathogens. A qualitative DNA determination of periodontal pathogenic microorganisms (Porphyromonas gingivalis, Prevotella intermedia, Actinobacillus actinomycetemcomitans, Treponema denticola, Bacteroides forsythus) was performed with polymerase chain reaction (PCR). Specific plots of DNA were amplified with DNA amplifier "Tertsyk" ("DNA Technology", Moscow) using specific multiplex oligonucleotide primers from "MultyDent" kit ("Gentech", Russia). DNA identification was performed by electrophoresis in 2% agarose gels stained with ethidium bromide, followed by visualization under UV light and photography. The obtained DNA solution was used to further quantify the biota in the samples, performed with a multiplex PCR on a real-time detecting amplifier DT-322 ("DNA Technology", Moscow) using "Femoflor" kit ("DNA Technology"), which allows to determine such factors as the total bacterial mass and microorganisms/groups of microorganisms: Lactobacillus sp., Enterobacteriaceae, Streptococcus sp., Staphylococcus sp., Gardnerella vaginalis / Prevotella bivia / Porphyromonas sp., Eubacterium sp., Sneathia sp. / Leptotrihia sp. /

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Fusobacterium sp., Megasphaera sp. / Veillonella sp. / Dialister sp., Lachnobacterium sp. / Clostridium sp., Mobiluncus sp. / Corynebacterium sp., Peptostreptococcus sp., Atopobium (At.) vaginae, Mycoplasma (M.) (genitalium + hominis), Ureaplasma (urealyticum + parvum), Candida sp. The polymorphic site of the Asp299Gly TLR4 gene was amplified by PCR using specific oligonucleotide primers.

Statistical analysis of the data was performed with a standard software package «STATISTICA 6.0» (StatSoft, Inc., USA). For data comparisons nonparametric methods were used: Wilcoxon test, t-test, intragroup correlations and covariances. Differences were considered statistically significant at P<0,05. Comparison of genotype frequencies between the groups was performed by analysis of 3x2 combination tables with Fisher's exact test. To compare frequencies of variants in unrelated groups we calculated the odds ratio (OR) with 95% confidence interval (CI).

Results and discussion

According to the research goal we studied tissue samples of autopsy material of coronary arteries of 31 patients who died of IHD, and of 5 patients who died of other causes and did not have lesions of coronary arteries. An analysis of the 5 tissue samples of coronary arteries of the patients who died of causes unrelated to atherosclerosis showed no microorganisms in the vascular wall.

An analysis for presence of major parodontopathogenic microorganisms in the atherosclerosis plague tissues of the coronary arteries revealed presence of at least one of the microorganisms in 26 of 31 patients, which comprised 83,9%. The most frequently found microorganisms were P. gingivalis - 64,5%, T. denticola - 41,9%, A. actinomycetemcomitans - 32,3%, while B. forsythus i P. intermedia were found less frequently - 12,9% i 6,5% respectively. Notably, presence of three microorganisms was found in 22,6% samples. The prevalent microbial associations were comprised of P. gingivalis, T. denticola, A. actinomycetemcomitans.

In the next stage of our research we studied frequency of the Asp299Gly gene TLR4 among individuals of Poltava population (n = 100) and among the patients who died of IHD (n = 31). The distribution frequency of polymorphic variants of the TLR4 gene corresponded to the Hardy-Weinberg principle (Pearson $\chi 2$ with Yates correction was 0,79 i 2,67 respectively). A comparison of genotype frequencies (Table 1) demonstrated a tendency to increased frequencies of the genotypes AG and GG in the group of patients who died of IHD (p = 0,086). We found the investigated groups of the microorganisms except At. vaginae, M. hominis and M. genitalium in all autopsy samples. We also observed that the allele G was significantly more frequently found (p = 0,04) in the subjects who died of IHD, the odds ratio was 2,92 (1,15-7,41) with 95% confidence interval.

Table 1

The distribution of genotypes and allele frequencies of gene polymorphisms TLR4 Asp299Gly among Poltava population died of coronary heart disease (CHD), % (n)

The gene, polymorphism	The frequency of genotype	Group population control (n=100)	Group deaths from coronary heart disease (n=31)	p*	The frequency of genotype	Group population control	Group deaths from coronary heart disease	χ^2 Pearson, df=1	RR (95% CI)	p**
TLR4 Asp299Gly	AA	90 (90)	77,42 (24)	0,086	A	94,5 (189)	85,5 (53)	4,25	2,92 (1,15-7,41)	0,04
	AG	9 (9)	16,13 (5)							
	GG	1 (1)	6,45 (2)		G	5,5 (11)	14,5 (9)			

p - the level of significance obtained Fisher's exact test*

*p** - the level of significance of the resulting test χ^2*

We found 15 with the genotype AA (Asp299Asp) gene TLR4, 3 - with the genotype AG and 2 - with the genotype GG in the biopsy material. Because of low frequency of the allel G, both AG and GG genotypes were combined into one group as carriers of the allele G.

According to the obtained results (Table 2) the carriers of the allele G had a significantly higher content of microbial DNA of the groups of Lactobacillus sp. - 4,20 ± 0,62 vs 3,01 ± 0,14 in the carriers of the allele A (AA) (p < 0,05), Enterobacterium sp. - 4,68 ± 0,87 vs 3,09 ± 0,17 (p < 0,05), Sneatia sp. / Leptotrichia sp. / Fusobacterium sp. - 3,47 ± 0,60 vs 1,78 ± 0,16 (p < 0,05), Mobiluncus sp. / Corynebacterium sp. - 3,06 ± 0,46 vs 2,18 ± 0,10 (p < 0,05), Peptostreptococcus sp. - 3,08 ± 0,67 vs 2,00 ± 0,11 (p < 0,05). To clarify the possible microbial associations and their relationship to the presence or absence of polymorphic variants of TLR4 intragroup correlations were investigated. Among the carriers of the allele A (AA genotype) positive significant (p < 0.05) correlations were found between the number of gene-equivalents of the pairs: Lactobacillus sp. - Enterobacterium sp. (0,984877); Lactobacillus sp. -Staphylococcus sp. (0,781613); Lactobacillus sp. -Eubacterium sp. (0,554622); Lactobacillus sp. -Mobiluncus sp. / Corynebacterium sp. (0,773681); Enterobacterium sp. - Staphylococcus sp. (0,793504) i Enterobacterium sp. - Mobiluncus sp. / Corynebacterium sp. (0,771553); Staphylococcus sp. -Eubacterium sp. (0,697129) and Staphylococcus sp. -Mobiluncus sp. / Corynebacterium sp. (0,613646). Among the carriers of the allele G (genotypes AG and GG) statistically significant positive correlations were also observed, except for an absence of the pair Lactobacillus sp. - Eubacterium sp. and an emergence of a new pair: Lachnobacterium sp. / Clostridium sp. -Lactobacillus sp. (0,826326); Lachnobacterium sp. / Clostridium sp. - Enterobacterium sp. (0,854616); Lachnobacterium sp. / Clostridium sp. -Staphylococcus sp. (0,831228) i Lachnobacterium sp. / Clostridium sp. - Mobiluncus sp. / Corynebacterium sp. (0,909823).

Table 2
The number of gene-equivalent groups of microorganisms isolated from atherosclerotic altered coronary arteries of patients with different genotypes of the gene Toll-like receptor 4 (TLR4)

Genotype	Lactobacillus spp.	Enterobacteriaceae	Streptococcus spp.	Staphylococcus spp.	Gardnerella vaginalis/Prevotella bivia/Porphyromonas spp.	Eubacterium spp.	Sneathia spp./Leptotrihia spp./Fusobacterium spp.	Megasphaera spp./Veillonella spp./Dialister spp.	Lachnobacterium spp./Clostridium spp.	Mobiluncus spp./Corynebacterium spp.	Peptostreptococcus spp.	Ureaplasma (urealyticum+parvum)	Candida spp.
AA (n=15)	3.01± 0.14	3.09± 0.17	2.38± 0.11	2.44± 0.13	2.82± 0.19	2.84± 0.26	1.78± 0.16	2.08± 0.21	3.48± 1.06	2.18± 0.10	2.00± 0.11	1.74± 0.13	3.03± 0.05
AG+GG (n=5)	**4.20± 0.62***	**4.68± 0.87***	2.96± 0.33	3.18± 0.64	3.50± 0.71	3.24± 0.45	**3.47± 0.60***	2.7± 0.45	2.9± 0.36	**3.06± 0.46***	**3.08± 0.67***	1.76± 0.10	3.14± 0.10

*Note: * - the significance of differences between genotypes p<0,05.*

Our data on the presence of microbial DNA in tissues of atherosclerotic plaques is confirmed in studies by other authors. Previously, we have obtained evidence that TLR4 Asp299Gly gene polymorphism determines the possibility of infection with the most common urogenital infections [1], rheumatoid arthritis [2]. It was reported that an analysis of 454 tissue samples of atherosclerotic plaques as well as samples of aortic and intestinal microbiota revealed presence of Chryseomonas in all samples and that of Veillonella and Streptococcus in most plaque samples (which correlated with their presence in the mouth) [6]. It was shown that P. gingivalis plays an important role in aortic intimal hyperplasia in patients with periodontitis [5]. Our data of increased contamination of atherosclerotic tissue by microorganisms in patients with polymorphic allele TLR4 Gly 299 can be explained by a reduced signal transmission ability of this receptor form, caused in particular by Gram-negative bacteria [4]. These findings support a possible involvement of these groups of microorganisms in the pathogenesis of atherosclerosis and the role of a polymorphic variant of the allele G of the TLR4 gene in an increase of microbial contamination of coronary artery tissues. The presence of a polymorphic variant of the allele G of the TLR4 gene in the genotype of individuals with atherosclerotic coronary arteries determines an increased contamination of the atherosclerotic plaque tissue by the following microorganisms: Lactobacillus sp., Enterobacterium sp., Sneathia sp. / Leptotrihia sp. / Fusobacterium sp., Mobilincus sp. / Corynebacterium sp., Peptostreptococcus sp.

Conclusions

Thus, from our findings we can conclude that a polymorphism of the TLR4 gene is of importance in the pathogenesis of coronary atherosclerosis and development of IHD. The risk of developing IHD is 2,92 bigger in the individuals who carry the allele 299Gly compared to those with the allele 299Asp. Therefore, it is necessary to continue research of the TLR4 gene polymorphism for a more detailed understanding of the genetic structure of susceptibility to IHD, which will bring us closer to understanding the mechanisms of interaction between a genetic variation of TLR and initiation of atherogenesis. At the same time, some microorganisms, including periodontal pathogens P. gingivalis, T. denticola, A. actinomycetemcomitans, B. forsythus and P. intermedia may also play an important role in the pathogenesis of IHD. It evokes a need to further study the subject further as well as identify the infection sources, their elimination and finding new effective schemes of possible use of antibiotics in the treatment of IHD.

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