Immunoglobulin in Atherosclerotic Lesions of Human Aorta

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SUMMARY

An augmented expression of mRNA for IgG light chain was demonstrated age-dependently on atheromatous lesions of the aorta in Watanabe heritable hyperlipidemic (WHHL) rabbits. The present study was designed to determine factors related to inflammation in human vessels excised during surgery. We detected IgG mRNAs using RT-PCR in human atherosclerotic lesions but not in human umbilical arteries which have no atheromatous lesions. To determine the clonality of IgGs, cDNAs encoding variable regions of IgG heavy chain were examined using RT-PCR. Atherosclerotic lesions had several subtypes of IgG gene families' suggesting the involvement of polyclonal B-cells. mRNAs of interleukins-6 (IL-6), -1α (IL- 1α), and -1β (IL- 1β) were also detected in the same samples. In summary, inflammatory reactions were present in the atherosclerosis lesion. (Jpn Heart J 1999; 40: 765-773)

Key words: Atherosclerosis, IgG subtypes, Interleukins, Polyclonal B-cell

ATHEROSCLEROSIS is one of the most serious health problems in modernized countries. Atherosclerotic lesions are characterized by the accumulation of lipids, leukocytes such as macrophages and monocytes, smooth muscle cells, and connective tissues at the vascular intima. Several mechanisms have been proposed to explain how human aortas are injured.¹⁻³⁾ One of the well known theories for the pathogenesis of atherosclerosis is the "response to injury" hypothesis proposed by Ross.¹⁾ Numerous mediators in the formation have been proposed, including growth factors, cytokines and vasoreactive molecules. However, the mechanisms that trigger the endothelial injury and that mediate the proliferation of intimal cells and subsequent accumulation of lipids, macrophages or other cellular components of arteries are still controversial.¹⁾

In the previous study, it was revealed that mRNA for IgG light chain was expressed exclusively in atherosclerotic lesions of WHHL rabbit aorta.⁴⁾ In a preliminary study in WHHL rabbits, the arterial contents of IgG increased progressively with age (not published). Recently, both a chronic inflammatory sign

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and focal immune responses were demonstrated in plaque lesions in human atherosclerosis. Virus and/or bacteria infections have been a focus of attention as candidates for the initiative pathogens,^{2,3} however, their causality remains controversial.⁵

The goal of the present study was to detect expressions of IgG and cytokine mRNAs in human aortic samples and to define the clonality of mRNA of IgG heavy chain.

MATERIALS AND METHODS

Reagents: All restriction enzymes, T4 DNA polymerase, T4 polynucleotide kinase, calf intestine alkaline phosphatase, reverse transcriptase, and Taq polymerase were obtained from Takara Shuzo (Kyoto, Japan); and oligonucleotides from Pharmacia Biotech (Buckingham, UK).

Vascular specimens: Fresh samples of human ascending aorta were taken during surgery from patients ($n = 9$; aged, 55–74 years old) on whom coronary artery by-pass graft (CABG) had been performed (see Table I). The aortic samples were immediately washed with sterile saline and placed on dry ice. Aortic specimens were frozen in O.C.T. compound (Miles, USA) and sliced into 3–5 μm thickness. These thin sections were stained with hematoxylin and eosin. The severity of the atherosclerosis was assessed according to the definition of the American Heart Association (AHA).⁶

Umbilical arteries and veins were prepared from fresh umbilical cords ($n = 2$). Residues of Warton jelly were removed and both arteries and vein were washed with sterile saline.

All subjects enrolled in the study gave informed consent. The study protocol was in agreement with the guidelines of the ethics committee at our institution.

Reverse transcriptase — polymerase chain reaction (RT-PCR): Total RNAs were extracted from atherosclerotic aorta and umbilical vessels using the guanidium thiocyanate/CsCl method.⁷ cDNAs were synthesized from 5 μg of total RNAs using an oligo (dT) 11–18 primer and 40 units of reverse transcriptase (RAV-2), in 10 μl of reverse transcription buffer (50 mmol/l Tris-HCl, pH 8.3, 75 mmol/l KCl, 3 mmol/l MgCl_2 , 10 mmol/l DTT, 0.2% Nonidet P-40 and 0.5 mmol/l dNTP) at 42°C for 1 hour.

The poly (dT) tailed cDNAs were amplified with TaKaRa Ex Taq polymerase, and oligonucleotides were constructed as follows. 1) To analyze the variable region of human IgG heavy chain in human aorta, the 5' end primers of 6 major groups (VH1–VH6) were constructed from the leader sequences of human IgG heavy chain.⁸ The primer sequences are shown in Table IIA. The 3' end of the primers were constructed from conserved sequences within the con-

stant regions (CH 1) of the heavy chains (see Table IIA).⁹⁾ 2) Primers specific for human interleukin-6 (IL-6) cDNAs were synthesized (see Table IIB).¹⁰⁾ 3) Human interleukin-1 α , 1 β (IL-1 α , IL-1 β) primers^{11,12)} are shown in Table IIB. 4) As an internal control, primers specific for human β -actin were synthesized¹³⁾; the sense primer sequence was (5'-TGTGCTATCCCTGTACG-CCTCT-3') and that of the antisense primer was (5'-TGAACCTTTGGGGGA-TGCTCGCT-3').

Polymerase chain reaction (PCR): Two μ l of first-strand cDNAs were amplified in PCR buffer containing 10 pmol of each primer and 0.25 units of TaKaRa Ex Taq DNA polymerase in 25 μ l of standard PCR buffer (10 mmol/l Tris-HCl, pH 8.8, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.1% Triton X-100 and 0.25 mmol/l each dNTP). Perkin Elmer Gene Amp PCR System 2400 (California, USA) was used with the following profile for amplification of human heavy chain variable region: 94°C for 1 minute, 50°C for 2 minutes and 72°C for 3

Table I. Patients' Profiles

Pt	Age	Sex	HT	DM	HL	Smoking	AHA class
A	72	f	-	-	+	-	IIa
B	71	m	+	-	-	-	IIa
C	71	m	+	-	-	-	IIa
D	63	m	+	+	-	-	IIa
E	68	m	-	-	+	+	III
F	63	m	+	+	-	-	III
G	60	m	+	-	-	+	IV
H	55	m	-	-	+	-	V
I	74	m	-	-	-	+	V

Pt = patients; f = female; m = male; HT = hypertension; DM = diabetes; HL = hyperlipidemia. AHA class (IIa, III, IV, V) showing the severity of atherosclerotic lesion assessed according to a definition of the American Heart Association.

Table II.

A: Design of Oligonucleotide Primers, Corresponding to Immunoglobulin Leader Sequences

Human IgG heavy chain	
VH1	5'-ATGGACTGGACCTGGAGG-3'
VH2	5'-ATACTTTGTTCCACGCTCCT-3'
VH3	5'-GAGTTTGGGCTGAGCTGG-3'
VH4	5'-CTGGTGCCAGCTCCCAGA-3'
VH5	5'-ATCCTCGCCCTCCTCCTG-3'
VH6	5'-TGTCTCCTTCCTCATCTTCC-3'
CH1	5'-ATCAGGAAGTGGTCCGTCGG-3'

(ref 8, 9)

B: Design of Oligonucleotide Primers, Corresponding to Interleukin Sequences

Human interleukins	Sense primer	Antisense primer
IL-6	5'-AGAAGCTCATCTCCCCTCCA-3'	5'-ACAATCTGAGGTGCCCATGCTA-3'
IL-1 α	5'-CTGTCTCTGAATCAGAAATCC-3'	5'-CATCACAGGTAGTGAGACC-3'
IL-1 β	5'-TTCCAGGACCTGGACCTCT-3'	5'-TCCCAGGAAGACGGGCAT-3'

minutes, for 45 cycles.^{9,14} The profiles for amplification of human IL-6, IL-1 α , IL-1 β , and β -actin were 94°C for 30 seconds, 54°C for 1 minute and 72°C for 2 minutes, for 25 or 30 cycles. PCR products were loaded onto 2% agarose gels, stained with ethidium bromide and separated.

NIH digital imaging analysis: Quantitative image analysis of the PCR products on the gel was performed on a Power Macintosh 8100 Computer using the public domain NIH image program (written by Wayne Rasband at the US National Institutes of Health and available via the Internet by anonymous FTP from zippy.nimh.nih.gov) with the macros Gel Plotting Andreas.txt (written by Andreas Becker and available from the same site in the directory pub/nih-image/user-macros). The procedure is described extensively in the manual "About Gel Macros" (gel macros.doc.hqx).^{15,16}

Data analysis: The sum of pixel values of each single line was measured within the selected boundaries. Every count of PCR products was standardized by dividing by the count of products for β -actin.

RESULTS

Genotypic variability of human IgG heavy chain: The patients' profiles are

Figure 1. IgG heavy chain PCR products derived from first-strand cDNAs. Total RNAs (5 μ g) prepared from human aorta were extracted and amplified as described in Materials and Methods. Ten μ l from a 25 μ l PCR reaction were applied to 2% agarose gel stained with ethidium bromide for electrophoresis. This figure shows the PCR products of 9 patients (lane A-I) and lane Um shows products of umbilical artery for each primer. Grades of the atherosclerosis were determined by American Heart Association (AHA) classification.

Table III. RT-PCR Analysis

Sample	VH1	VH2	VH3	VH4	VH5	VH6	IL6	IL1 α	IL1 β
A	ND	ND	ND	ND	+	+	+	+	+
B	ND	+	ND	+	+	+	+	ND	+
C	+	+	+	ND	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+
E	ND	ND	ND	+	+	+	+	+	+
F	ND	ND	+	ND	+	+	+	ND	+
G	ND	ND	ND	ND	+	+	+	+	+
H	ND	ND	ND	+	+	+	+	ND	+
I	ND	+	ND	ND	+	ND	+	+	+
Um	ND	ND	ND	ND	ND	ND	ND	ND	ND

Amounts of PCR products were analyzed using an NIH image program. (+) showed detected cDNAs. ND was not detectable. A-I = patient's sample of atherosclerotic lesions; Um = umbilical cord.

Figure 2. Expression of cytokine mRNAs in human aorta. cDNAs of the following cytokines IL-6, IL-1 α , and IL-1 β were amplified in the present patients. β -actin was also amplified as an internal control. PCR products (10 μ l) were also loaded from 25 μ l PCR reaction. The levels of β -actin product were not different between the patients (aorta) and umbilical artery samples at both 25 cycle and 30 cycle amplification.

shown in Table I. To analyze the expression of IgG mRNAs in human aorta, cDNAs of variable regions were amplified using a specific constant region primer (CH 1) and six oligonucleotide primers (VH1–VH6) corresponding to the leader sequences encoding region as shown in Figure 1 and Table III. cDNAs corresponding to VH1 to VH6 primers were detected in 2, 4, 3, 4, 9 and 8 patients, respectively. Each patient had 2 to 6 types of IgG subtypes. There was no detectable mRNA of IgG heavy chain in human umbilical arteries.

Expression of cytokine mRNAs: cDNAs of human IL-6, IL-1 α and 1 β were amplified mostly in atherosclerotic aortas but not in human umbilical arteries

Figure 3. Photomicrographs of aorta sections. Representative photomicrographs of sections of thoracic aorta from patients who underwent CABG operation. A and C were stained with hematoxylin and eosin, whereas B and D were stained with elastic masson. Sections A and B or C and D were obtained from the same sample. Slices A and B showed adaptive intimal thickening. In slices C and D extracellular lipid forms and fibrous thickening were observed (type V). Thrombus was present at the surface of this lesion. Standard bars present 2 mm.

(Figure 2, Table III). There were no significant correlations between types of IgG heavy chain or cytokine and the levels of atherosclerosis in AHA classification.

Photomicrographs of the aorta sections: Thin slices of aortic samples are shown in Figure 3.

DISCUSSION

This study has demonstrated that mRNAs of immunoglobulins were expressed in atherosclerotic human aorta but not in human umbilical arteries, and that they were polyclonal. The presense of mRNAs for IgG and analysis of their clonality were the first observation in human vascular samples.

The expression of mRNAs of IgG heavy chain does not necessarily indicate the production of IgGs in atherosclerotic human aortas. However, in a preliminary study in WHHL rabbits, the aortic tissue contents of IgG and their quantities of mRNAs increased progressively with positive relativity (not published).

Kosaka *et al.* showed that the combination of VH3 and VH4 was genetically over-expressed in patients with autoimmune disease.⁸⁾ In the present study, various combinations of IgG subclasses were expressed in aortic samples of 9 patients examined. None of the 9 cases in the present study had any autoimmune disease. However, to elucidate the possibility of autoimmune processes in the evolution and progression of atherosclerosis, a specific factor for atherogenesis should be identified. The present existence of all subtypes of IgG including VH1–VH6 indicates polyclonality into B-cells, which migrated into human aorta.

mRNAs of IL-6 and IL-1 β were expressed at the atherosclerotic aorta in all 9 patients. IL-6 is well known as a B-cell differentiation factor that is secreted by various cells including lymphocytes, macrophages, fibroblasts, endothelial cells and smooth muscle cells. Since IL-6 promotes the proliferation of B lymphocytes, the maturation of B cells for plasma cells may occur at the atherosclerotic lesions.^{17,18)} Consequently, large amounts of IgGs are produced from plasma cells.⁴⁾

In general, once the cells constituting an artery are injured, macrophages produce IL-1 which stimulates other lymphocytes to produce other cytokines such as IL-6, TNF α , IL-8 and GM-CSF.¹⁹⁾ In this study, both IL-6 and IL-1 were expressed at the atherosclerotic lesions in human aorta, which indicates the occurrence of local inflammatory and/or immunological reactions. It is also considered that in atherosclerotic lesions the secretion of cytokines and production of IgG molecules result in initial inflammation. Nevertheless, the trigger of inflammation is still unknown.

In the current study, the presence of large amounts of IgG and cytokines suggest the occurrence of an immunological reaction in atherosclerotic lesions. However, expression patterns of mRNAs for IgG heavy chain and/or interleukins were not related to risk factors such as hypertension, diabetes, smoking habits, hyperlipidemia or severity of atherosclerosis. It could not be concluded that these findings contributed to causing the vascular injury, residual products or results of aging. If the presence of IgGs causes the development of vascular damage, the antigen against IgGs detected in this study remains unknown. The presence of IgGs suggests the existence of antigens. With regard to identifying chemically the evolved antigens, some candidates have been already reported, including oxidized LDL and chlamydia pneumoniae in atherosclerotic lesions.^{20,21)} These findings suggest the occurrence of an immunological reaction in atherosclerotic lesions, however, the antigen-antibody interactions remain to be elucidated.

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