ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(6): 417–422 **CN** 31-1940/Q

Effect of Ozone Produced from Antibody-catalyzed Water Oxidation on Pathogenesis of Atherosclerosis

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Abstract Recent studies have suggested that antibodies can catalyze the generation of unknown oxidants including hydrogen peroxide (H_2O_2) and ozone (O_3) from singlet oxygen ($^1O_2$) and water. This study is aimed to detect the effect of antibody-catalyzed water oxidation on atherosclerosis. Our results showed that both H_2O_2 and O_3 were produced in human leukemia THP-1 monocytes incubated with human immunoglobulin G and phorbol myristate acetate. In the THP-1 monocytes incubated with human immunoglobulin G, phorbol myristate acetate and low density lipoprotein, the intracellular total cholesterol, free cholesterol, cholesteryl ester and lipid peroxides clearly increased, and a larger number of foam cells were observed by oil red O staining. The accumulation of all intracellular lipids was significantly inhibited by vinylbenzoic acid, and only slightly affected by catalase. These findings suggested that the production of O_3, rather than H_2O_2, might be involved in the pathogenesis of atherosclerosis through the antibody-catalyzed water oxidation pathway.

Key words antibody; ozone; THP-1 monocyte; foam cell; atherosclerosis

Antibodies are classical adaptor molecules of the immune system. In terms of the antibody effector mechanism, the central idea is that antibodies do not have destructive abilities, but mark foreign antigens and pathogens for removal by complement cascade and/or phagocytosis [1]. However, the recent discovery of a new property of antibody molecules suggests a previously unexplored effector function of the immune system. All immunoglobulins (Ig), regardless of source or antigenic specificity, can catalyze the reaction between singlet oxygen ($^1O_2$) and water to facilitate the production of a number of water oxidants, such as hydrogen peroxide (H_2O_2) and ozone (O_3), which are highly bioreactive and cytotoxic compounds, resulting in direct killing of foreign antigens or pathogens [2–5]. The discovery of this pathway greatly enriches our knowledge of the ability of antibodies to destroy foreign pathogens. However, it also suggests that there might be some potential pathophysiologic effects in this antibody-mediated activity. O_3 is a kind of gas with high chemical reactivity, and H_2O_2 is a strong oxidant. As well as the efficient killing of pathogens, they might also lead to oxidative damage on tissues.

Atherosclerosis has been considered a chronic inflammatory disease, in which the oxidative damage of lipids and the consequent formation of foam cells are key steps in onset and development [6]. It is now widely accepted that the inflammation associated with the presence of leukocytes and antibodies is a crucial component in human atherosclerosis [7]. It has been suggested that H_2O_2, as a strong oxidant, is an important risk factor for atherosclerosis, and recent research also reported that the O_3 oxidation products of cholesterol triggered the foam cell formation in tissue macrophages [8].

Can the antibody-catalyzed water oxidation pathway, however, produce H_2O_2 and O_3 at the same time, both of which play major roles in the generation and development of atherosclerosis? And what impact does H_2O_2 or O_3

Received: December 12, 2005 Accepted: April 5, 2006
This work was supported by the grants from the Science and Technology Key Program of the Science and Technology Department of Sichuan Province (No. 02SY029-123) and the Ph.D. Programs Foundation of the Ministry of Education of China (No. 20050610050)
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DOI: 10.1111/j.1745-7270.2006.00181.x

production in this pathway impose on the pathogenesis of atherosclerosis? In this study, we investigated the production of H_2O_2 and O_3 by the antibody-catalyzed water oxidation pathway, and their effects on intracellular accumulation of cholesterol and lipid peroxides in human leukemia THP-1 monocytes and on foam cell formation. The clarification of these questions will provide valuable information for better understanding of atherosclerosis, as well as for searching for new ways to prevent and treat this disease.

Materials and Methods

Materials

Human leukemia cell line THP-1 has been widely used as a model for monocyte-macrophage lineage. THP-1 monocytes were provided by the Department of Immunology, Sichuan University (Chengdu, China). Human IgG was purchased from the Chinese Academy of Medical Sciences (Beijing, China). Vinylbenzoic acid was from Fluka (Buchs, Switzerland). Sodium cholate was from Amresco (Solon, USA). Malonic dialdehyde assay kit was from Jiancheng Bioengineering Institute (Nanjing, China). RPMI 1640 medium was from Gibco (Carlsbad, USA). Bovine serum albumin, phorbol myristate acetate (PMA), oil red O, indigo carmine, bovine catalase, cholesterol oxidase, cholesteryl ester (CE) hydrolase, p-hydroxyphenylacetic acid and horseradish peroxidase (HRP) were from Sigma-Aldrich (St. Louis, USA).

Preparation of low density lipoprotein (LDL)

Isolation of human LDL was carried out using NaBr gradient ultracentrifugation [9], followed by 48 h dialysis against phosphate-buffered saline (PBS) containing 200 μM EDTA at 4 ºC. LDL was then passed through a 0.22 μm sterilized millipore filter, followed by an agarose gel (0.5%) electrophoresis to identify the purity. The protein content of LDL was determined using Coomassie brilliant blue G-250 staining.

Cell culture

The THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (56 ºC, 45 min), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 ºC in 5% CO_2. The well-cultured cells were washed three times with PBS, and shaken for 10 min each time to remove the IgG bound nonspecifically. Except for the analysis of H_2O_2 and O_3, cell concentration was adjusted to 1×10^6 cells/ml in RPMI 1640 medium supplemented with 0.2% bovine serum albumin, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were then divided into seven groups: research group, 0.5 μg/ml PMA, 100 μg/ml LDL, 1 mg/ml human IgG in cultured cells; catalase group, supplementing 100 U/ml (final concentration) bovine catalase to the research group; vinylbenzoic acid goup, supplementing 0.1 mM (final concentration) vinylbenzoic acid to the research goup; control group 1, no treatment in cultured cells; control group 2, only supplementing LDL in cultured cells; control group 3, omitting human IgG from the research group; control group 4, omitting PMA from the research group. Each group was cultured for 48 h before analyzing the content of cholesterol and lipid peroxides in cells, and the formation of foam cells.

Analysis of accumulation of cholesterol and content of lipid peroxides in cells

The cultured THP-1 monocytes were collected by scraping using a cell scraper, and washed three times with PBS. After re-suspension in 0.5 ml of sodium phosphate buffer solution (0.1 M, pH 7.4), each sample was subjected to ultrasonication for 1 min using the microtip of a sonifier. Samples (0.1 ml) were used for the measurement of cell protein using Coomassie brilliant blue G-250 staining, and the remainder of the samples was for the measurement of cell-associated cholesterol and lipid peroxides.

For total cholesterol (TC) determination, as described previously [10], 0.1 ml of the sample was added to 0.9 ml of assay solution (0.1 U/ml cholesterol oxidase, 1 U/ml HRP, 0.01 U/ml CE hydrolase, 0.05% Triton X-100, 1 mM sodium cholate and 0.6 mg/ml p-hydroxyphenylacetic acid in 0.1 M sodium phosphate buffer solution, pH 7.4) and incubated at 37 ºC for 1 h. Fluorescence was measured in a spectrofluorophotometer (excitation, 325 nm; emission, 415 nm). For determination of free cholesterol (FC), CE hydrolase was omitted from the assay solution. CE was determined by substraction of FC from TC.

The content of lipid peroxides was determined by estimating malonic dialdehyde using the thiobarbituric acid method with an assay kit.

Oil red O staining for foam cells

The cultured THP-1 monocytes were washed with PBS three times and fixed in 2.5% glutardialdehyde for 3 h, dipped in 2.5% potassium dichromate for 16 h, and stained in 1% oil red O for 20 min to identify lipid droplets in cytoplasm. Cell nuclei were then stained in hematoxylin for a few seconds. For each procedure, the fixed cells

were washed with distilled H_2O. THP-1 monocyte-derived foam cells were observed and photographed. Semi-quantitative analysis of foam cells was evaluated by the percentage of positive oil red O-staining cells.

Analysis of H_2O_2

After removing the IgG bound nonspecifically, the cultured THP-1 monocytes were collected and diluted to 1.5×10^7 cells/ml in PBS containing 1 mg/ml human IgG. After the cells were incubated at 4 ºC for 1 h, they were activated at 37 ºC for 10 min by the addition of PMA (1 μg/ml). Every aliquot (1 ml) was removed from the cell suspension at 3 min intervals and filtered through a 0.22 μm syringe. Cell filtrates were collected at each time point during the reaction, and the H_2O_2 content of each filtrate was monitored by the following method [11]: 2.6 ml of Tris-HCl buffer solution (pH 7.4, 0.1 M), 0.1 ml of 2.0 U/ml HRP solution and 0.1 ml of cell filtrate were added to 0.2 ml of p-hydroxyphenylacetic acid solution (7.5 mM). One minute later, fluorescence was measured by a spectrofluorophotometer (excitation, 325 nm; emission, 415 nm). Two control groups were set: the cells of one group were not activated by PMA, and the cell suspension of another group did not contain human IgG.

Analysis of indigo carmine oxidation during THP-1 monocyte activation

After removing the IgG bound nonspecifically, the cultured THP-1 monocytes were collected and diluted to 1.5×10^7 cells/ml in PBS containing 1 mg/ml human IgG. After the cells were incubated at 4 ºC for 1 h, they were activated at 37 ºC for 10 min by the addition of PMA (1 μg/ml). As described previously, a solution of the O_3 probe, indigo carmine, in PBS (pH 7.4) was added to the activated cells to give a final concentration of 30 μM. Every aliquot (1 ml) was removed from the cell suspension at 3 min intervals and filtered through a 0.22 μm syringe. Four comparing-control groups were set: group 1, supplementing a final concentration of 100 U/ml bovine catalase in the cell suspension; group 2, supplementing a final concentration of 0.1 mM vinylbenzoic acid in the cell suspension; group 3, no PMA in the cell suspension; group 4, no IgG in the cell suspension. The indigo carmine content at each time point was determined by a spectrophotometer at 610 nm [12].

Statistical analysis

Data were analyzed using SPSS 11.0 for Windows (SPSS, Chicago, USA) and presented as mean±SD. For comparison between multiple groups, quantitative data were analyzed using one-way ANOVA and the least significant difference test, and qualitative data were analyzed using the χ^2-test. *P* value less than 0.05 was considered significant.

Results

Effect of antibody on H_2O_2 production by activated THP-1 monocytes

When human IgG-coated THP-1 monocytes were activated by PMA, the production of H_2O_2 was significantly increased as time progressed. In the absence of IgG, the production of H_2O_2 by PMA-activated THP-1 monocytes was much lower than by IgG-coated THP-1 monocytes activated with PMA (**Fig. 1**).

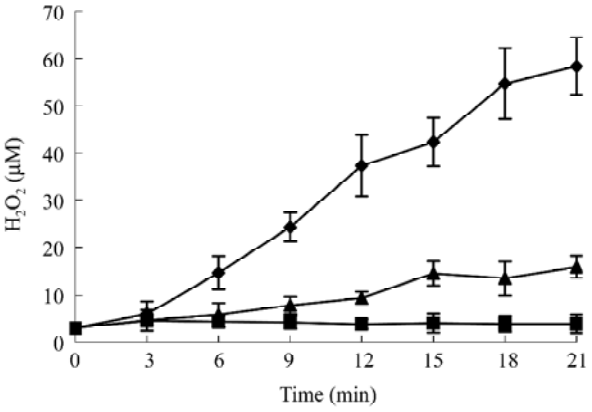

Fig. 1 H_2O_2 produced by activated human leukemia THP-1 monocytes

◆, immunoglobulin (Ig) G-coated THP-1 monocytes activated with phorbol myristate acetate (PMA); ▲, PMA-activated THP-1 monocytes without IgG; ■, unactivated THP-1 monocytes with IgG.

Effect of antibody on O_3 production by activated THP-1 monocytes

The antibody-catalyzed O_3 generation was assayed by indigo carmine bleaching reaction, a sensitive chemical probe for O_3. When human IgG-coated THP-1 monocytes were activated by PMA, the indigo carmine was markedly bleached, which was slightly enhanced by catalase and significantly inhibited by vinylbenzoic acid. Omitting PMA or IgG produced no bleaching reaction of indigo carmine (**Fig. 2**).

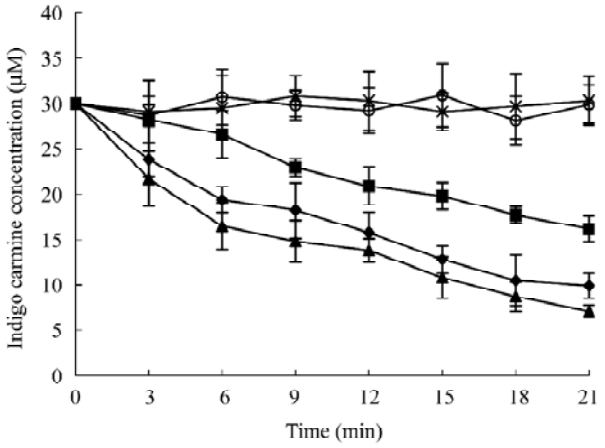

Fig. 2 Oxidation of indigo carmine by activated human leukemia THP-1 monocytes

◆, immunoglobulin (Ig)G-coated THP-1 monocytes activated with phorbol myristate acetate (PMA); ▲, IgG-coated THP-1 monocytes activated with PMA and catalase; ■, IgG-coated THP-1 monocytes activated with PMA and vinylbenzoic acid; ×, PMA-activated THP-1 monocytes without IgG; ○, IgG-coated THP-1 monocytes activated without PMA.

Effect of antibody on accumulation of cholesterol and lipid peroxides in activated THP-1 monocytes

When THP-1 monocytes were co-cultured with LDL, PMA and IgG, all the intracellular lipids, including TC, FC, CE and lipid peroxides, increased significantly compared with control groups ($P<0.01$ or $P<0.05$). The above parameters were clearly inhibited by vinylbenzoic acid ($P<0.01$ or $P<0.05$), and only slightly affected by catalase (**Table 1**).

Effect of antibody on foam cell formation from activated THP-1 monocytes

When THP-1 monocytes were co-cultured with LDL, PMA, and IgG, a great number of foam cells were found by oil red O staining (**Fig. 3**), and the percentage of foam cells was much higher than that of control groups ($P<0.01$). This process was also significantly inhibited by vinylbenzoic acid ($P<0.05$), and only slightly affected by catalase (**Table 2**).

Discussion

It was considered that the generation of O_3 and H_2O_2 by antibody-catalyzed water oxidation needs the participation of $^1O_2$ [3–4], as the following equation:

$$H_2O + 2O_2 \xrightarrow{IgG} H_2O_2 + O_3$$

If the antibody-mediated oxidation has any significant

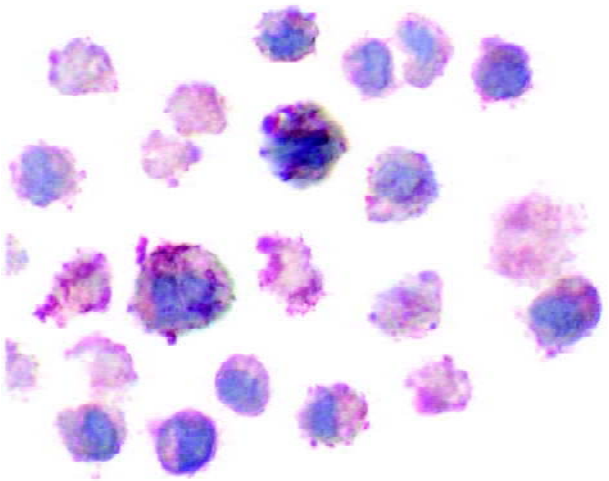

Fig. 3 Photographs of positive oil red O-staining human leukemia THP-1 cells (magnification, 400×)

Table 1 Content of cholesterol and lipid peroxides in human leukemia THP-1 monocytes

Group	TC [μg·(mg protein)$^{-1}$]	FC [μg·(mg protein)$^{-1}$]	CE [μg·(mg protein)$^{-1}$]	MDA [nmol·(mg protein)$^{-1}$]	*n*
No treatment	13.6±1.3*	10.2±1.9*	3.4±0.6*	0.43±0.07*	6
LDL	14.6±1.6*	10.9±1.8*	3.7±0.6*	0.40±0.04*	6
PMA+LDL	31.0±3.4*	21.1±2.9#	10.1±1.4*	1.92±0.46*	6
LDL+IgG	16.8±3.1*	11.0±1.8*	5.8±1.3*	0.6±0.08*	6
PMA+LDL+IgG	98.2±8.5	36.6±6.9	61.6±6.1	8.50±1.23	6
PMA+LDL+IgG+catalase	101.2±9.3	36.4±5.4	64.8±6.8	8.65±1.17	6
PMA+LDL+IgG+vinylbenzoic acid	57.3±6.1*	28.7±3.1#	29.7±4.9*	4.95±0.74#	6

*$P<0.01$, #$P<0.05$, compared with the phorbol myristate acetate (PMA)+low density lipoprotein (LDL)+immunoglobulin (Ig)G group. CE, cholesteryl ester; FC, free cholesterol; MDA, malonic dialdehyde; TC, total cholesterol.

Table 2 Percentage of positive oil red O-staining cells in human leukemia THP-1 monocytes

Group	Positive cells (%)	*n*
No treatment	4.8±1.5 *	6
LDL	4.7±2.2 *	6
PMA+LDL	16.3±3.4 *	6
LDL+IgG	6.8±1.9 *	6
PMA+LDL+IgG	74.0±6.4	6
PMA+LDL+IgG+catalase	77.8±7.0	6
PMA+LDL+IgG+vinylbenzoic acid	46.2±5.8 #	6

*$P<0.01$, #$P<0.05$, compared with the phorbol myristate acetate (PMA)+low density lipoprotein (LDL)+immunoglobulin (Ig) G group.

role *in vivo*, what is the origin of the high-energy $^1O_2$ molecule that is required for initiation of the pathway? The primary source of $^1O_2$ *in vivo* is thought to be activated phagocytes, including monocytes and neutrophils [13,14]. The amount of $^1O_2$ generated during the neutrophil respiratory burst has been estimated to be up to 19% of the total oxygen consumption of the respiratory burst [15]. Furthermore, the monocytes/macrophages highly express high-affinity IgG receptors on their surface [16], which can bind a great deal of IgG. Given that monocytes/macrophages have the potential both to produce $^1O_2$ and to bind antibodies, we consider that these cells could be a biological source of O_3 and H_2O_2. We report here further analytical evidence that antibody-coated THP-1 monocytes, after activation with PMA, produce both O_3 and H_2O_2, thus supporting this hypothesis.

In this study, we detected O_3 production from THP-1 monocytes using the indigo carmine bleaching reaction. It is important to reiterate here that indigo carmine, although a sensitive probe for O_3 detection [3], is not very specific, because $^1O_2$ can also bleach indigo carmine. So vinylbenzoic acid, a more specific ozone trap [12], was used for distinguishing the possible involvement of $^1O_2$ in the bleaching reaction. Our results showed that the indigo carmine bleaching reaction was significantly inhibited by vinylbenzoic acid, which offered strong evidence that O_3 was indeed produced in this reaction system. By adding catalase to the systerm, the indigo carmine bleaching reaction was enhanced slightly. It is likely that H_2O_2 was removed by catalase, which would prolong the lifetime of O_3 and enhance the O_3 signature, as H_2O_2 has been found to decompose O_3 through a peroxone process [17].

Our study further showed that PMA-activated THP-1 monocytes, when cultured with IgG and LDL, were clearly transformed into foam cells, and the contents of intracellular TC, FC, CE and lipid peroxides in THP-1 monocytes increased significantly. It has been proposed that foam cell formation is the result of internalization of oxidation-modified LDL (Ox-LDL) by monocyte-derived macrophages through scavenger receptors on the surfaces of these cells. The internalization leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, resulting in the formation of foam cells [18]. Therefore, our findings implied that antibody-catalyzed water oxidation could have participated in the oxidative modification of LDL and the formation of macrophage-derived foam cells. This would be of profound significance to the pathogenesis of atherosclerosis, because it is now widely believed that the foam cell formation by Ox-LDL is a major pathologic characteristic of atherosclerosis [19]. The accumulation of foam cells leads to the formation of fatty streak lesions, which is a critical event in the early stages of atherosclerosis. In the advanced stages of atherosclerosis, the death and necrosis of foam cells facilitates the development of vulnerable atherosclerotic plaques with large lipid cores and very thin fibrous caps, the rupture of which leads to thrombus formation followed by clinical manifestations of coronary heart disease, such as myocardial infarction [20]. Thus, the foam cell formation represents a key event in the development and progression of atherosclerosis. The known biological activities of Ox-LDL, in addition to its role in the formation of foam cells, also include: the chemotactic activity for circulating monocytes, a cytotoxic effect on endothelial cells; the induction in endothelial cells of several factors such as granulocyte macrophage colony-stimulating factor, cell adhesion molecules, platelet-derived growth factor and heparin-binding epidermal growth factor-like protein; the induction of platelet-derived growth factor in smooth muscle cells; the impairment of endothelium-dependent arterial relaxation; and the stimulation of platelet aggregation [21]. All of these events are involved in the development and exacerbation of atherosclerosis. Taken together, our work provided novel insights into the pathogenetic role of antibody-catalyzed water oxidation in atherosclerosis.

The antibody-coated THP-1 monocytes, after activation with PMA, can produce large amounts of O_3 and H_2O_2, both of which are strong oxidants, and might induce the oxidative modification of LDL. Thus, they might be involved in the process of intracellular accumulation of lipids and foam cell formation. However, which one is the main factor in the oxidative damage of lipids and the formation of foam cells? This study showed that the intracellular accumulation of TC, FC, CE and lipid

peroxides, and the formation of foam cells induced by antibody-catalyzed water oxidation, were significantly inhibited by vinylbenzoic acid, a specific ozone trap. In contrast, the above parameters were hardly affected by catalase, which can remove H_2O_2. This finding suggests the involvement of O_3 in the oxidative damage of lipids and the formation of foam cells. H_2O_2 seems to have an imperceptible effect on the process.

In conclusion, these results suggested that the production of O_3, not H_2O_2, through the antibody-catalyzed water oxidation pathway, could be an important mechanism for the pathogenesis and development of atherosclerosis. The recent evidence of O_3 formation in human atherosclerotic arteries reported by Wentworth *et al.* [8] provides strong support for this probability. Further study *in vivo* is necessary to elucidate the positive evidence of the endogenous production of O_3 through the antibody-catalyzed water oxidation pathway. As well as a highly chemically reactive character, O_3 also elicits the production of cytokines, including tumor necrosis factor-α and interleukin-8 [22], thereby allowing the amplification of the inflammatory cascade, which is a contributory factor in atherosclerosis.

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Edited by
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