

USE OF A MONOCLONAL ANTIBODY TO DETECT APOLIPOPROTEIN B IN CUPRIC SULFATE OXIDIZED LOW DENSITY LIPOPROTEINS AND IN HUMAN SERUM

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ABSTRACT

A sensitive sandwich enzyme linked immunosorbent assay (ELISA) was used to detect changes undergone by low density lipoproteins (LDL) when oxidized in the presence of SO₄Cu. The monoclonal antibody used as the capture antibody in the assay recognized chemical modifications in apolipoprotein B (Apo B) at early stages of the oxidative process. Using this ELISA, measurable amounts of modified Apo B were detected in sera from 61 patients with lower limb obliterans atherosclerosis and in 61 healthy individuals. The patients had higher levels than the control group (55 ± 19 and 28 ± 13 UM Apo B/dL, respectively, $p < 0.0001$). A significant correlation ($r = 0.86$, $p < 0.001$) was found between the levels of modified Apo B and those of lipoperoxides. We conclude that this method might be useful for investigating oxidized LDL in human serum as an early predictor of atherosclerosis.

Key words: oxidized low density lipoproteins, LDL, monoclonal antibody, MAb, enzyme-linked immunosorbent assay, ELISA, atherosclerosis

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RESUMEN

Se utilizó un ensayo inmunoenzimático en fase sólida (ELISA) para detectar cambios en las lipoproteínas de baja densidad cuando son oxidadas en presencia de sulfato cúprico. El anticuerpo monoclonal, empleado como anticuerpo de captura en el ensayo, reconoce modificaciones químicas que se producen en la apolipoproteína B (Apo B) durante los primeros estadios del proceso oxidativo. Con este ELISA se detectaron niveles significativamente superiores de Apo B modificada en el suero de pacientes con aterosclerosis periférica en relación con individuos normales utilizados como grupo control (55 ± 19 y 28 ± 13 UM Apo B/dL, respectivamente, $p < 0,0001$). Los niveles de Apo B modificada se correlacionaron significativamente con los niveles de peróxidos lipídicos ($r = 0,86$; $p < 0,001$). Se concluye que este método puede ser útil para la detección de LDL oxidada en suero humano como indicador precoz de riesgo aterogénico.

Palabras claves: lipoproteínas de baja densidad oxidadas, LDL, anticuerpo monoclonal, AcM, ensayo inmunoenzimático en fase sólida, ELISA, aterosclerosis

Introduction

It has been suggested that the oxidative modified low density lipoprotein (O-LDL) plays an important role in the atherosclerotic process (1, 2). O-LDL is taken up readily by macrophages leading to the formation of lipid-laden foam cells, similar to the characteristic cell type of the early atherosclerotic lesion (3). Although there is convincing evidence of the presence of O-LDL in the subendothelial space (4), the possibility of an oxidative modification of LDL in the blood stream remains controversial.

Recently, we obtained a monoclonal antibody (MAb) that recognizes an epitope that is created or exposed in the apolipoprotein B (Apo B) molecule during the oxidative modification of LDL (5). We now report the use of this MAb (IA/CB VLDL-1) in the detection of the *in vitro* susceptibility of the LDL to be oxidized by copper ions and in the detection of oxidized LDL in human sera.

Materials and Methods

LDL ($1.019 < d < 1.063$) were isolated from pooled plasma of healthy persons (1 mg EDTA/mL blood) by ultracentrifugation (6). The concentration of LDL is given in terms of its protein content (7). Before the treatment with cupric sulfate (SO₄Cu), the LDL was dialyzed at 4 °C for 18 h against phosphate buffer saline (PBS), pH 7.4, to remove EDTA and sterilized through a 0.2 µm filter.

Isolated LDL was incubated at a concentration of 200 µg/mL in PBS with 10 mM SO₄Cu during 3.5 h at 37 °C in a 6-cell holder spectrophotometer (UV-160 Shimadzu). A control of native LDL without the SO₄Cu treatment was maintained in the same conditions. Samples of LDL were taken at different times of incubation and the reaction was stopped by cooling the samples at -20 °C, immediately after the addition of EDTA to a final concentration of 50 µM.

The lipid peroxidation process was monitored by measuring the thiobarbituric acid reactive substance

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(TBARS) (8), the formation of conjugated dienes (9) and the modification of the Apo B molecule by an ELISA based on MAb IA/CB VLDL-1. Details of the ELISA were described elsewhere (5). Briefly, polystyrene ELISA plates (Maxisorb, Nunc) were coated with the MAb at 10 µg/mL in a bicarbonate buffer, pH 9.6, at 4 °C overnight. After blocking the plate with PBS-1 % skim milk, duplicate samples of LDL (diluted 1:20) at different times of incubation with SO_4Cu were added to the wells and incubated for 1 hour at 25 °C. After washing, a polyclonal antibody against human Apo B, labeled with horseradish peroxidase, was added to the wells and incubated for 1 h at 25 °C. After a new washing, orthophenyldiamine (0.04 %) and H_2O_2 (0.015 %), in citrate-phosphate buffer, pH 5.0, were added to the wells as the substrate. The reaction was stopped after 20 min by adding 2.5 M SO_4H_2 and the absorbance at 492 nm measured in a microplate reader. For total Apo B measurement in the samples the MAb for coating the ELISA plates was substituted by the anti-Apo B polyclonal antibody.

In order to measure modified Apo B in fresh serum, as an indicator of oxidized LDL in the blood stream, blood samples were collected from 61 patients with clinical and angiographic criteria of obliterans atherosclerosis of the lower limbs, and 61 healthy volunteers, matched by age and sex, without any symptoms of cardiovascular diseases. Blood samples were allowed to clot at 25 °C for 1 h, centrifuged at 3000 rpm for 15 min and the sera was used immediately. Apo B was measured in the samples by the ELISA described above. A standard curve was drawn by using as the standard the SO_4Cu modified-LDL, prepared as described above. We assigned a value of 1 unit of "modified Apo B"/mL (UM-Apo B/mL) to a 1:50 dilution of this modified LDL (in PBS-bovine serum albumin 1 %). Serum samples were diluted 1:50 in the same buffer. One hundred microliters of samples and standards were added as duplicates in the wells and incubated 1 h at 25 °C. The ELISA continued as was described.

In the samples, the level of total and HDL-cholesterol, and triglycerides were assayed by enzymatic colorimetric methods, by the use of kits from Boehringer-Mannheim. LDL-cholesterol was estimated by the Friedwald formula (10), and apolipoprotein B and A1 were assayed by ELISA (11, 12). The level of lipoperoxides, as malondialdehyde (MDA), were also measured (13).

Results and Discussion

As shown in Figure 1, for conjugated dienes and modified Apo B, a time dependence, and a lag and propagation phases were observed. These results indicate that in LDL oxidation by Cu^{2+} there is a modification not only of the polyunsaturated fatty acids (PUFAs), but also of the Apo B molecules that can be detected by MAb IA/CB-VLDL1. In five inde-

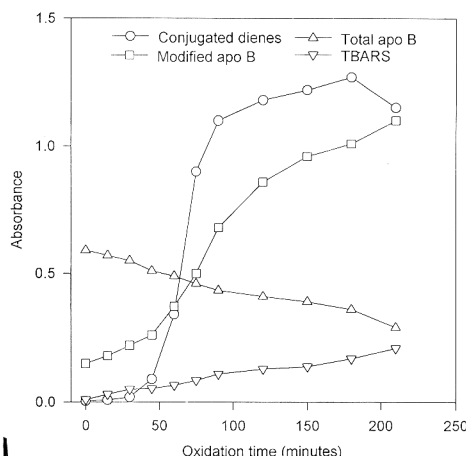


Figure 1. Absorbance values for conjugated dienes (234 nm), modified and total Apo B (492 nm) and TBARS (532 nm) in LDL oxidized with cupric sulfate.

pendent experiments a high correlation between the length of the lag phase for conjugated dienes and for the Apo B modification was found ($r = 0.988$; $p < 0.001$).

The total Apo B using the polyclonal antibody in the ELISA, showed lower values at higher oxidation times (Figure 1), probably as a consequence of the fragmentation of the Apo B molecule. This indicates that the epitope detected in Apo B by MAb IA/CB-VLDL1 in SO_4Cu treated LDL appears before the fragmentation of the molecule observed in the later steps of the LDL oxidative process.

In order to determine if this modified Apo B is present in the blood stream, we studied fresh serum samples from patients with lower limbs obliterans atherosclerosis and healthy individuals. We found significantly higher values in patients than in the controls, 55 ± 19 and 28 ± 13 UM Apo B/dL, respectively, $p < 0.0001$ (Table 1). A distribution analysis showed that MAb IA/CB VLDL-1 discriminates patients from healthy controls very well (Figure 2).

Although the patients had a higher concentration of total Apo B than the control group, the difference in the level of modified Apo B between both groups can not be explained only as a result of this difference, because the ratio: modified Apo B/total Apo B was also significantly higher in the patients (Table 1). The level of lipoperoxides were also higher in patients and a significant correlation was found between lipoperoxides and the level of modified Apo B ($r = 0.86$; $p < 0.01$). Patients also had significantly higher levels of total cholesterol, LDL-cholesterol, and triglycerides than the control (Table 1).

The presence of anti-oxidized LDL antibodies in serum of patients with carotid and obliterans athero-

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Table 1. Lipids, lipoproteins, apolipoproteins and lipoperoxides in patients with peripheral atherosclerosis and a group of control subjects.

	Periph. atherosc. (n = 61) Mean (SD)	Control group (n = 61) Mean (SD)	p*
Age	57.8 (9.2)	58.3 (9.0)	0.41
Total cholest. (mmol/L)	6.81 (1.76)	5.89 (1.03)	0.007
LDL-cholest. (mmol/L)	4.98 (1.60)	4.22 (0.97)	0.04
HDL-cholest. (mmol/L)	1.02 (0.31)	1.13 (0.30)	0.63
Triglycerides (mmol/L)	1.45 (0.90)	1.08 (0.49)	0.002
Apo A1 (mg/dL)	148 (25.4)	152 (31.3)	0.65
Total Apo B (mg/dL)	117 (30.8)	105 (29.2)	0.04
Modified Apo B (UM ApoB/dL)	55 (19)	28 (13)	0.0001
Modified Apo B/total Apo B	0.56 (0.33)	0.29 (0.17)	0.001
Lipoperoxides (mmol MDA/L)	3.91 (0.68)	3.23 (0.62)	0.0001

*: Statistical significance, student t test.

sclerosis has been reported (14, 15). Most efforts devoted to the production of MAbs for the detection of oxidative modifications of lipoproteins have been based on the immunization with *in vitro* altered LDL, mainly with malonaldehyde (MDA-LDL). When these MAbs were used as capture antibodies in sandwich ELISAs, they recognized MDA-LDL and not normal LDL (16, 17), but also other MDA-proteins and LDL oxidized by Cu 2+ (16, 18). On the other hand, when MAb IA/CB-VLDL1 was

used as the capture antibody, in a similar sandwich ELISA, it recognizes only MDA-LDL or MDA-VLDL, and also LDL modified by lipoxigenase or endothelial cell culture, but not other MDA-proteins or lipoproteins (5). It means that the epitope recognized by this MAb is different from those reported with other MAbs. It is important to keep in mind that this MAb resulted from the fusion of lymphocytes derived from mice immunized with very low density lipoproteins (VLDL), purified from plasma of patients with peripheral atherosclerosis, where Apo B antigenic determinants, altered by lipidic peroxidation, could be present. Then, the modified lipoproteins detected by MAb IA-CB VLDL1 may be of clinical relevance.

Now we have found that the Apo B modification detected by this MAb, induced by an exogenous oxidative stress with SO₄Cu, occurs before an evident Apo B molecule fragmentation. This result indicates that the new or unmasked epitope detected is exposed in the Apo B molecule during the early steps of the LDL oxidative modification.

Because it has been proposed that minimally or mildly modified LDL plays an important role in the pathogenesis of human atherosclerosis (19), the high level of modified Apo B detected by MAb IA/CB-VLDL1 in sera of patients with obliterans atherosclerosis, suggests the use of this MAb for investigating oxidized LDL in the human blood stream as an early predictor of the atherosclerotic process.

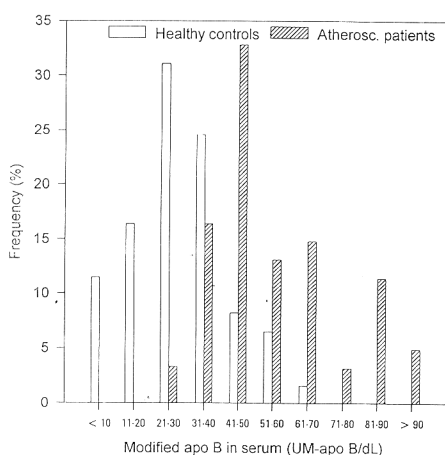


Figure 2. Frequency distribution of modified Apo B in serum from healthy controls and patients with peripheral atherosclerosis.

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