ORIGINAL RESEARCH

Susceptibility to apoptosis of lymphocytes from patients with peripheral arterial disease

Katarzyna Skórkowska-Telichowska MD, PhD^{1,2}

Rajmund Adamiec MD, PhD¹

Dominika Tuchendler MD³

Kazimierz Gąsiorowski MD, PhD⁴

¹ Clinic of Angiology, Hypertension, and Diabetology, Wrocław Medical University, Poland

² Endocrinology Department, Military Hospital, Wrocław, Poland

³ Cardiology Department, Military Hospital, Wrocław, Poland

⁴ Department of Basic Medical Sciences, Wrocław Medical University, Poland

Manuscript submitted 17th January, 2009

Manuscript accepted 11th August, 2009

Clin Invest Med 2009; 32 (5): E345-E351.

Abstract

Purpose. To determine, *in vitro*, the susceptibility to apoptosis of lymphocytes from patients with peripheral arterial disease (PAD) in the presence of a low culture medium serum concentration, and to evaluate the correlation of the degree of apoptosis and the serum lipid levels.

Methods. Lymphocytes were isolated from the venous blood of PAD patients with lower limb ischemia secondary to obliterative atherosclerosis of Fontaine stage IIb. None of the patients had received hypo-lipemic therapy. The lymphocytes were incubated for 48 hr in media containing reduced concentrations of fetal calf serum. The study group consisted of 10 patients (7 men and 3 women), with a mean age of 67.0 ± 4.0 yr. The control group consisted of ten healthy volunteers, of the same mean age and sex proportion as the study group.

Results. The percentage of non-apoptotic lymphocytes was lower (by 17%) and the percentage of late apoptotic lymphocytes was higher (by 33%) in the PAD patients than in the healthy donors when comparing the slopes of regression lines describing the relation between frequency of apoptotic lymphocytes in culture media containing reduced concentration of fetal calf serum. The percentage of late apoptotic lymphocytes was correlated with the levels of total cholesterol ($r_s=0.93$; $P<0.01$) and LDL cholesterol

($r_s=0.80$; $P<0.01$), and negatively correlated with the level of triglycerides ($r_s=-0.71$; $P<0.05$).

Conclusion. The results of this study of lymphocyte apoptosis are important in understanding of the disease pathogenesis and should be taken into account in elaboration of treatment strategies.

Peripheral arterial disease (PAD) is an advanced form of systemic atherosclerosis that is associated with increased cardiovascular morbidity and mortality. Research in humans and in animals indicates that atherosclerosis can be considered a chronic inflammatory response.^{1,2}

An important mechanism in the pathology of the disease is apoptosis.³⁻⁵ Apoptosis can occur in all of the cell types found in atheromatous plaques, including endothelial cells, smooth muscle cells, macrophages and lymphocytes. The proportion of apoptotic cells in an atheromatous plaque depends strongly on the stage of plaque development. It is especially high in advanced lesions, where 30 - 60% of cells show

TABLE 1. Selected information on the patients included in the study.

Patient	Sex	Age yr	BMI kg/m ²	TC mg%	HDL mg%	LDL mg%	TG mg%	ABI right	ABI left	walking distance - m
1	F	64	31	189	24	140	128	1.1	0.4	148
2	F	60	28	205	44	138	124	1.0	0.5	135
3	F	67	20	229	60	145	123	0.7	0.6	87
4	M	70	29	177	52	91	170	0.7	1.1	138
5	M	69	31	215	31	146	193	0.8	0.7	181
6	M	63	35	142	29	60	269	0.5	0.8	126
7	M	64	24	149	30	95	125	0.8	0.6	178
8	M	72	29	213	42	138	177	1.0	0.7	128
9	M	73	30	265	19	107	695	1.0	0.8	142
10	M	66	28	212	29	143	200	0.8	0.9	154

morphological features consistent with apoptosis. It plays a major role in the destabilization and rupture of atherosclerotic plaques and promotes thrombogenesis at the sites of damage to the blood vessel walls.^{4,5}

Earlier studies indicated that peripheral lymphocytes from patients with PAD may be more susceptible to apoptosis than those from healthy donors.⁶ The aim of this study was to determine how the susceptibility to apoptosis of lymphocytes from patients with PAD is affected by serum deprivation, *in vitro*, and whether this correlates with the serum lipid concentrations.

Materials and Methods

Patients

The study was approved by the local bioethics committee. All of the patients were provided with written information on the purpose and design of the study. The study group consisted of 10 patients (7 men, 3 women) of the Wrocław Medical University Clinic of Angiology, Hypertension and Diabetology. All were suffering from advanced peripheral arterial disease (PAD), and none had received hypo-lipemic therapy. Their mean age was 67.0 ± 4.0 yr. The subjects had been diagnosed with lower limb ischemia secondary to obliterative atherosclerosis of Fountain stage IIb. The time since the onset of symptoms was at least five years in all cases. The control group consisted of 10

healthy volunteers and had the same mean age and sex proportion as the study group.

PAD was diagnosed using a Sonodop 4000 DSM 2P Doppler segmental sphygmometer (Sonoteknik GmbH). A walking distance was also performed using a TrackMaster set at 3.2 km/h with a slope of 12%. Routine laboratory tests were performed in all subjects to assess their general state of health. Peripheral blood samples were collected in the morning after an overnight fast. All patients had abnormal lipid profiles. Some had a normal total cholesterol level, but a low HDL level or a high triglyceride level. Selected information on the subjects is presented in Table 1.

Isolation and incubation of lymphocytes

Peripheral blood samples were collected in tubes containing heparin at the same time that the samples for routine testing were taken. Mononuclear cells were isolated by density gradient centrifugation with Histo-paque 1077 (Sigma, St. Louis, MO, USA). Cell viability was tested using trypan blue.

The lymphocytes were counted and suspended in Eagle's medium to a final cell density of 0.5×10^6 /ml. All media were supplemented with 10 µg/ml mitogenic lectin PHA-M and 25 µg/ml gentamycin. The media were also supplemented with fetal calf serum (FCS) to the following concentrations: 10, 5, 2.5, 1.25 and 0.625%. The lymphocytes were then incubated for 48 hr at 37°C in a CO₂ incubator.

Detection and quantification of apoptosis

At the end of the incubation period, apoptotic cells were detected and quantified using an APO-AF apoptosis detection kit (Sigma, St. Louis, MO, USA). Briefly, the suspension was centrifuged, and the cells were resuspended and stained with Annexin V-FITC/PI for 15 min at room temperature in the dark. Then, 50 - 80 μ l of the suspension were placed on a glass slide and covered with a cover slip. The lymphocytes were examined under a Nikon Eclipse E-600 fluorescence microscope using two filter blocks: B-2A (annexin-FITC) and G-2A (PI).

1,000 lymphocytes per sample were examined at random and categorized according to the staining, as follows:

- non-apoptotic: unstained;
- early apoptotic: green fluorescence;
- late apoptotic: halo of green fluorescence with a red nucleus; and
- necrotic: uniform red fluorescence.

Statistical analysis

For each patient, regression equations were calculated for the percentage of lymphocytes at each stage of apoptosis and the concentrations of FCS in the medium. The coefficient b from each regression equation ($Y = a + bx$) was then used to compare the slopes of the regression lines in PAD patients group with those in healthy people.⁷ We calculated Spearman's rank correlation (r_s) between b and the main lipid fractions of the lipid profile for all 10 patients. The statistical significance of r_s was estimated from table of critical values of Spearman's rank correlation coefficient. Finally, the determination coefficients were calculated using the formula: $d = r_s^2$.

The calculations were carried out using the routine statistical methods.⁷

Results

The percentages of non-apoptotic and late apoptotic lymphocytes after 48 hr culture with a lower concentration of FCS were different in patients with PAD from the results in healthy blood donors. Based on the results of the ANOVA test, the differences between the patients and the healthy donors were significant for both non-apoptotic lymphocytes ($F = 8.22$, $df = 1$, $P = 0.009$) and late apoptotic lymphocytes ($F = 7.46$, $df = 1$, $P = 0.013$). The percentages of early apoptotic and necrotic lymphocytes did not differ between patients and healthy donors.

The frequencies of non-apoptotic and late apoptotic lymphocytes in 48-hour cultures in relation to the concentrations of FCS in the cell culture medium were calculated in regression equations. Figure 1 shows the regression equations and regression lines for the representation of non-apoptotic and late apoptotic lymphocytes in cultures of cells derived from the venous blood of healthy donors and PAD patients.

The percentage of non-apoptotic lymphocytes was similar in patients and healthy donors at higher concentrations of FCS, but was different at lower concentration of FCS. The slope of the regression line was steeper for patients than for healthy donors, and the coefficient b of the regression equation was 17% higher for patients than for healthy donors (Fig. 1A).

The percentage of late apoptotic lymphocytes increased with decreasing concentrations of FCS. The slope of the regression line was again steeper for the patients than for the healthy donors, and the coefficient b of the regression equation was about 33% higher for the patients than for the healthy donors. At the lowest concentration of FCS tested, the percentage of late apoptotic lymphocytes was 30% higher for the patients than for the healthy donors (Fig. 1B).

The coefficient b from each regression equation was then used to calculate the Spearman's rank correlation. The correlation coefficients and determination coefficients for the levels of each lipid fraction and the coefficient b are presented in Table 2.

FIGURE 1. Figure 1 A, B The concentration of fetal calf serum (FCS) in the medium and the percentage of non-apoptotic viable lymphocytes (1A) and late apoptotic lymphocytes (1B) in PAD patients and in healthy donors.

TABLE 2. The correlation coefficients (r_s) between the total cholesterol (TC), HDL cholesterol, LDL cholesterol and triglycerides (TG) and lymphocyte % at each stage of apoptosis in the study group.

Cells:	TC			HDL			LDL			TG		
	r_s	d	P	r_s	d	P	r_s	d	P	r_s	d	P
Non-apoptotic	-0.87	0.76	<0.01	-0.59	0.35	>0.05 (NS)	-0.48	0.23	>0.05 (NS)	0.36	0.13	>0.05 (NS)
Early apoptotic	0.65	0.42	0.05	0.48	0.23	>0.05 (NS)	0.30	0.09	>0.05 (NS)	-0.11	0.01	>0.05 (NS)
Late apoptotic	0.93	0.86	<0.01	0.29	0.08	>0.05 (NS)	0.80	0.64	<0.01	-0.71	0.50	<0.05
Necrotic	-0.85	0.72	<0.01	-0.32	0.10	>0.05 (NS)	-0.68	0.46	<0.05	0.53	0.28	>0.05 (NS)

The percentage of non-apoptotic lymphocytes was negatively correlated with the total cholesterol level. The percentage of late apoptotic lymphocytes was positively correlated with the total cholesterol and LDL cholesterol levels, and negatively correlated with the triglyceride level. The percentage of necrotic lymphocytes was negatively correlated with the total cholesterol level.

Determination coefficients > 0.49, given in bold in Table 2, indicate that the compared data are strongly correlated, and that the observed variability in compared data influence each other.⁷ For instance, the determination coefficient: $d=0.86$ calculated with the correlation coefficient between total cholesterol level and late-apoptotic cells frequency suggests that 86% of observed variability in total cholesterol level in examined PAD patients could be interpreted as strongly affecting variations of late apoptotic cell frequency in those patients. The determination coefficients and the correlation coefficients of total cholesterol level and necrotic cell number and of total cholesterol and non-apoptotic cell number indicate that elevated cholesterol levels were responsible for 76% of the decreased level of non-apoptotic lymphocytes, and for 72% of the reduced frequency of necrotic lymphocytes. The higher the cholesterol level, the lower the non-apoptotic cells and necrotic cells and the higher late apoptotic cell numbers.

Discussion

Lymphocytes of patients with PAD were more susceptible to apoptosis *in vitro* when the concentration of FCS in the medium was less than the optimum concentration of 10%. Serum deprivation is a useful technique for determining the susceptibility of cells to apoptosis, since the viability of cells in culture depends on the content of growth factors and other activators of signal transduction pathways in the medium. Serum deprivation reduces the levels of these factors, thereby inducing apoptosis.⁸

In our study, the level of HDL cholesterol did not correlate with the percentages of early apoptotic lymphocytes or late apoptotic lymphocytes cultured at low concentrations of FCS. This does not concur with the results of a recent study in which an increased level of HDL cholesterol decreased the susceptibility of cells to apoptosis.⁹ This discrepancy may be due to the patients in our study having lipid profiles indicative of advanced atherosclerosis, with low HDL levels that may have had only a slight effect on lymphocyte susceptibility to apoptosis.

The level of triglycerides correlated with the percentage of non-apoptotic lymphocytes, and negatively correlated with the percentage of late apoptotic lymphocytes. It has been documented that the effect of triglycerides on leukocyte apoptosis is complex, and depends strongly on fatty acid composition. Saturated fatty acids promote apoptosis, whereas unsaturated fatty acids prevent apoptosis.^{10,11} In the context of the literature, we assume that unsaturated fatty acids pre-

vailed in the triglyceride composition of the serum blood lipids of our patients.

There was a strong correlation between the percentage of late apoptotic lymphocytes and total cholesterol and LDL cholesterol. Negative correlations between LDL cholesterol and total cholesterol and the percentage of non-apoptotic lymphocytes were also found. It has been documented previously that, in cell cultures, LDL cholesterol makes vascular cells more susceptible to apoptosis by inducing over-expression of Fas, FasL and Bax, and by reducing the expression of Bcl-2, and inhibiting that of NF- κ B.¹²⁻¹⁴ Interestingly, it was documented experimentally that serum from patients with acute coronary syndromes (ACS) triggered apoptosis in human endothelial cells, which supports the theory that circulating serum mediators induce apoptosis in atheromatous plaques, force an apoptosis susceptibility phenotype in blood cells and, finally, lead to plaque disruption.¹⁵ In the light of the literature, we suggest that a high level of LDL cholesterol in patients with PAD could also increase the susceptibility of peripheral lymphocytes to apoptosis. It is recognised that peripheral lymphocytes, including T lymphocytes, constantly enter the arterial intima during all stages of plaque formation.^{15,16} When the endothelium is activated by atherogenic factors, adhesion molecules (vascular cell adhesion molecule-1, intercellular adhesion molecule-1) become over-expressed and, with atheromatous chemo-attractants (oxLDL, monocyte chemoattractant protein-1), recruit blood derived cells (monocytes and T cells) to the plaques.^{1,17} Our results indicate that lymphocytes derived from the peripheral blood of patients with PAD are more susceptible to apoptosis, which suggests that atheromatous plaques are constantly entered by prone-to-apoptosis lymphocytes. By enhancing apoptosis in a plaque, residing - T lymphocytes may have beneficial effects on plaque stability¹⁶, since fewer T cells within a plaque could lead to a decrease in the local production and release of T cell-derived cytokines, among them IFN- γ and IL-2. These cytokines are able to activate macrophages in a plaque, and promote de-

struction of collagen within the fibrous cap of a plaque.¹⁶ Thus, the higher frequency of apoptosis in plaque lymphocytes could lead to a lower level of IFN- γ , making the plaques more stable. Strategies for treating atheromatosis based on the induction of apoptosis in the lymphocytes in atheromatous plaques merit further study.

References

1. Ross R: Atherosclerosis - an inflammatory disease. *N Engl J Med* 2005;340:115-26.
2. Frostegard J, Ulfgren A-K, Nyberg P et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 1999;145:33-43.
3. Geng Y-J, Libby P. Progression of atheroma. Struggle between death and procreation. *Arterioscl Thromb Vasc Biol* 2002;22:1370-80.
4. Kockx MM, Herman AG. Apoptosis in atherosclerosis: beneficial or detrimental? *Cardiovasc Res* 2000;45:736-46.
5. Guevara NV, Chen K-H, Chan L. Apoptosis in atherosclerosis: pathological and pharmacological implications. *Pharmacol Res* 2001;44:59-71.
6. Skórkowska-Telichowska K: PhD Thesis, Medical University, Wrocław, 2006.
7. Bourke G.J., Daly L.E., McGilvray J.: Interpretation and uses of medical statistics. (eds.: G.J. Bourke, L.E. Daly and J. McGilvray), Blackwell Scientific Publications, Oxford, London, Edinburgh, Boston, Palo Alto, Melbourne, 1985.
8. Kim Y H, Takahashi M, Suzuki E et al. Apoptosis induced by hydrogen peroxide under serum deprivation and its inhibition by anti-sense c-jun in F-MEL cells. *Biochem Biophys Res Commun* 2000;271:747-52.
9. Martinet W, Kockx. MM: Apoptosis in atherosclerosis: focus on oxidized lipids and inflammation. *Current Opin Lipidol* 2001;12:535-41.
10. Mishra R, Simonson MS. Saturated free fatty acids and apoptosis in microvascular mesangial cells: palmitate activates pro-apoptotic signaling involving caspase 9 and mitochondrial release of endonuclease G. *Cardiovasc Diabetol* 2005;4:1-12.
11. Listenberger LL, Han X, Lewis SE et al. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Nat Acad Sci* 2003;100:3077-82.

12. Salvayre R, Auge N, Benoist H et al. Oxidized low-density lipoprotein-induced apoptosis. *Biochim Biophys Acta* 2002;1585:213-21.
13. Kataoka H, Kume N, Miyamoto S et al. Oxidized LDL modulates Bax/Bcl-2 through the lectin-like oxLDL receptor -1 in vascular smooth muscle cells. *Atheroscler Thromb Vasc Biol* 2001;21:955-60.
14. Nishio E, Arimura S, Watanabe Y. Oxidized LDL induces apoptosis in cultured smooth muscle cells: a possible role for 7-ketocholesterol. *Biochem Biophys Res Commun* 1996;223:413-8.
15. Halvorsen B., Otterdal K., Dahl T. B. et al.: Atherosclerotic plaque stability – what determines fate of a plaque? *Prog Cardiovasc Dis* 2008;51:183-94.
16. de Boer OJ, Becker AE, van der Wal AC. T lymphocytes in atherogenesis – functional aspects and antigenic repertoire. *Cardiovasc Res* 2003;60:78-86.
17. Falk E.: Pathogenesis of atherosclerosis. *J Am Coll Cardiol* 2006, suppl.C: 7-12.

Correspondence to:

Katarzyna Skórkowska-Telichowska, M.D.
Endocrinology Department, Military Hospital,
R.Weigla 5 Str., 50-981 Wrocław, Poland
Phone: +48 71 76 60 667
Fax: +48 71 76 60 599
e-mail:cathcor@poczta.onet.pl