DOI 10.1007/s10517-015-3090-7

Cellular Composition of the Spleen and Changes in Splenic Lysosomes in the Dynamics of Dyslipidemia in Mice Caused by Repeated Administration of Poloxamer 407

N. V. Goncharova, A. V. Shurlygina, E. V. Mel'nikova, O. L. Karmatskikh, P. A. Avrorov, K. V. Loktev, and T. A. Korolenko

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 160, No. 7, pp. 34-37, July, 2015
Original article submitted April 10, 2014

We studied the effect of dyslipidemia induced by poloxamer 407 (300 mg/kg twice a week for 30 days) on cellular composition of the spleen and splenocyte lysosomes in mice. Changes in blood lipid profile included elevated concentrations of total cholesterol, aterogenic LDL, and triglycerides most pronounced in 24 h after the last poloxamer 407 injection; gradual normalization of lipid profile was observed in 4 days (except triglycerides) and 10 days. The most pronounced changes in the spleen (increase in organ weight and number of cells, inhibition in apoptosis, and reduced accumulation of vital dye acridine orange in lysosomes) were detected on day 4; on day 10, the indices returned to normal. Cathepsin D activity in the spleen also increased at these terms. The relationship between changes in the cellular composition of the spleen and dynamics of serum lipid profile in mice in dyslipidemia caused by repeated administrations of relatively low doses of poloxamer 407 is discussed.

Key Words: *dyslipidemia; poloxamer 407; spleen; lysosomes*

Administration of poloxamer 407 (P-407) is used as a new pharmacological model of hypercholesterolemia and triglyceridemia in rats and mice [7]. P-407 acts via inhibition of lipoprotein lipase followed by disturbance in catabolism of lipoproteins and their uptake by liver cells [1,6,7]. This model is characterized by a sharp increase in blood serum lipid indices in mice (with high doses of P-407: 500-1000 mg/kg once or repeatedly) far surpassing the corresponding parameters in humans with atherosclerosis [8,9]. The model of dyslipidemia in mice caused by repeated administration of relatively low doses of P-407 (300 mg/kg, twice a week during 30 days) is similar to changes in the lipid profile in dyslipoproteinemia of I, IV and V types in humans. Kupffer cells in the liver bind lipoproteins (and P-407), which leads to their enlargement and development of intracellular accumulation syndrome [7,8]. Similar changes probably occur in lysosomes of splenocytes and splenic macrophages. However, lysosomotropic properties of P-407 remain unknown. It can be assumed that lysosomes of different macrophage pools (liver, lungs and spleen) are the targets for P-407.

Here we studied the effect of P-407-induced dyslipidemia on cellular composition of the spleen and splenocyte lysosomes in mice.

MATERIALS AND METHODS

The study was carried out on male CBA/J mice (vivarium of Research Institute of Physiology and Basic Medicine) weighing 22-25 g, aged 2.5-3 months. P-407 (Pluronic F-127, Sigma) was administered in a dose of 300 mg/kg intraperitoneally twice a week for 30 days [7]. The animals were sacrificed 24 h after the last injection (expressed lipemia) and in 4 or 10 days after the last F-407 injection (gradual recovery from hyperlipidemia). Before decapitation, the animals

Research Institute of Physiology and Basic Medicine, Siberian Division of Russian Academy of Medical Sciences, Novosibirsk, Russia. ***Address for correspondence:*** t.a.korolenko@physiol.ru. T. A. Korolenko

0007-4888/15/1601-0028 © 2015 Springer Science+Business Media New York

were deprived of food for 14 h but had free access to water. Control mice were injected with corresponding volume of saline (0.25 ml).

The blood samples collected after decapitation were centrifuged (Eppendorf 5415R centrifuge) at 3000*g* for 15 min; the serum was stored at 70°C for no longer than 1 month and used for analytical studies.

Blood leukocyte count was assessed routinely in a Goryaev's chamber and expressed in mln/ml (10^6/ml). The concentrations of total cholesterol (TC) and triglycerides (TG), total protein, glucose, ALT and AST activity in blood serum were determined using Architect S8000 biochemical analyzer (Abbot), LDL-cholesterol was measured on a Labio 200 biochemical analyzer (Mindray) using Biosystems kits.

Spleen homogenates were prepared in a Potter–Elvehjem homogenizer in 0.25 M sucrose (pH 7.2). Cathepsin D activity was measured in spleen homogenates at pH 5.0 using 5 M urea-treated azocasein as the substrate (Fluka BioChemica) [9]. Triton X-100 was used for cell destruction (final concentration in the sample 0.1%).

The spleens were weighed and gently crushed in a glass homogenizer to prepare cell suspension. The cells were filtered, resuspended in RPMI-1640 medium, and used for calculation of their total number (in a Goryaev's chamber) in the organ and for cytofluorimetry. All manipulations with cell suspension were conducted at 0-4°C.

For analysis of lymphocyte subpopulations in the spleen, the cell suspension treated with rat anti-mouse monoclonal antibodies CD16/32 PE, CD4 PE, and CD8 FITC (BD Pharmingen) was analyzed on a FACSCalibur (BD) flow cytofluorometer equipped with Cell Quest software.

Quantification of cells in different cell cycle phases was carried out in cell suspension of lymphoid organs by flow cytometry using DNA-specific dye (PI) as described elsewhere [2,5] on a FACSCalibur cytofluorimeter (BD; Filter 585/42). The percentage of cells in the following cell cycle phases was determined: G0/G1 — resting phase (diploid, amount of DNA=2n); S and G2/M — synthetic, postsynthetic, and mitotic phases (hyperploid, DNA>2n), and hypodiploid cells (DNA<2n), most of which in apoptosis.

The cell suspension prepared from the spleen was incubated with acridine orange solution (5 μg/ml) in McIlvaine buffer pH 7.3 for 5 min. The dye was discharged, the cells were washed 3 times with RPMI-1640 medium, and a smear was prepared from the precipitate on a slide by dried droplet method, it was covered in phosphate buffered saline (pH 7.2-7.4) with a coverslip and examined under a LSM510 Meta confocal laser scanning microscope (Carl Zeiss).

The data was processed by nonparametric methods: Kruskal–Wallis rank ANOVA, Mann–Whitney test for intergroup comparisons, and Spearman's correlation analysis. Calculations were carried out using Statistica 6.0 software. The differences between means were considered significant at $p<0.05$.

RESULTS

Repeated treatment of mice with moderate doses of P-407 caused a sharp increase in concentration of TC ($p<0.001$), LDL-cholesterol ($p<0.001$), and especially TG ($p<0.001$) in 24 h (Table 1). Four days later these parameters decreased, with the exception of high TG concentrations ($p<0.05$), and 10 days later the lipid profile was completely normalized. Concentration of serum total protein increased in 24 h: 119.5±4.72 *vs*. 64.0±1.08 g/liter in the control ($p=0.0001$) probably due to P-407-induced sharp increase in lipoprotein level. ALT activity increased only 24 h after the last P-407 administration (50.00±4.67 U/ml *vs*. 37.80±4.45 U/liter in the control, $p<0.01$) and did not differ from the control at later terms (4 and 10 days). Glucose concentration and AST activity remained unchanged.

Nonparametric ANOVA showed an increase in spleen mass index (organ weight [mg]/body weight [g]) 24 h and 4 days after the last administration of P-407 (Table 2). Four days after repeated P-407 administration, the percentage of splenocytes expressing receptor Fc-γ ($CD16/32^+$) in the phase of apoptosis (hypodiploid cells) decreased (Table. 2), as well as leukocyte count (in 4 days, 1.15±0.08×10^6/ml; in 10 days,

TABLE 1. Effect of Repeated P-407 Administration on Lipid Profile of Mouse Blood Serum (*M*±*m*)

Group		TC, mmol/liter	LDL-cholesterol, mmol/liter	TG, mmol/liter
Control(*n*=20)		3.76±0.17	0.30±0.03	2.47±0.21
P-407	24 h (*n*=21)	10.2±0.31**	3.01±0.16**	37.39±2.22**
	4 days (*n*=9)	3.45±0.09	0.26±0.02	4.07±0.52*
	10 days (*n*=15)	3.73±0.06	0.24±0.01	2.96±0.19

Note. *$p<0.05$, **$p<0.001$ in comparison with the control.

TABLE 2. Spleen Weight Index and Cell Parameters at Different Observation Terms after P-407 Administration (*M*±*SE*)

Parameter	Control	In 24 h	In 4 days	In 10 days
Spleen weight index, mg/g	3.64±0.26	5.54±0.59*	5.26±0.33*	3.63±0.15
CD16/32$^+$, %	47.22±1.21	44.48±1.51	42.40±1.67*	43.98±2.27
Hypodiploid splenocytes (apoptosis), %	0.28±0.06	0.42±0.07	0.16±0.05*	0.30±0.06

Note. *$p<0.05$ in comparison with the control

0.67±0.09×10^6/ml; in the control, 2.26±0.37×10^6/ml, $p<0.05$). By day 10, the spleen mass index returned to normal values, as well as the level of CD16/32$^+$ cells, hypodiploid splenocytes (apoptosis) (Table 2). Administration of the corresponding volume of saline had no effect on the analyzed parameters.

In the spleen homogenate, activity of lysosomal enzyme cathepsin D increased in 24 h and 4 days (Fig. 1), which reflects increased proteolytic activity of spleen cells, probably due to macrophage migration.

Vital staining of isolated splenic cells (splenocytes and macrophages) witn acridine orange showed both reduced red and green fluorescence on days 4 and 10 (Fig. 2), which agreed with the data on reduced apoptosis in splenocytes and absence of oxidative stress in this model. This is indicative of decreased ability of lysosomes to accumulate the dye due to malfunction of the pump in their membrane and to lysosomal accumulation syndrome (lipids and probably P-407) [3].

In control animals (but not in P-407-treated mice), a positive correlation between red and green fluorescence of acridine orange (r_s=0.94, $p<0.05$) was found. This attested to impaired accumulation of acridine orange in lysosomes after exposure to P-407. High negative correlation between the number of cells expressing Fc-γ receptor, and cathepsin D activity (r_s=-0.98, p=0.000) on day 4 and a positive correlation on day 10 (r_s=0.83, p=0.041) were revealed. They reflected the dynamics of dye accumulation and changes in intra-lysosomal pH at different terms after exposure to P-407.

Thus, changes in the weight, cellular composition of the spleen, and lysosomal apparatus of splenocytes in this model occur in parallel with changes in the dynamics of lipid profile. This observation demonstrates the important role of P-407-induced lipemia in the functional state of peripheral immunity. However, unlike dyslipidemia of alimentary origin, there were no signs of splenocyte activation and oxidative stress in our model, as is evidenced by decreased apoptosis and number of cells expressing Fc-γ receptor. These results are consistent with reduced Fc-γ-dependent IL-6 production by splenocytes under the influence of P-407 described previously [12] and with data [15] on inhibition of inflammatory response in Kupffer cells and blockade of macrophage phagocytic activity by P-407 [10]. Perhaps, a similar mechanism works in the spleen, causing suppression of its immune function due to the blockade of macrophage-mediated immunity.

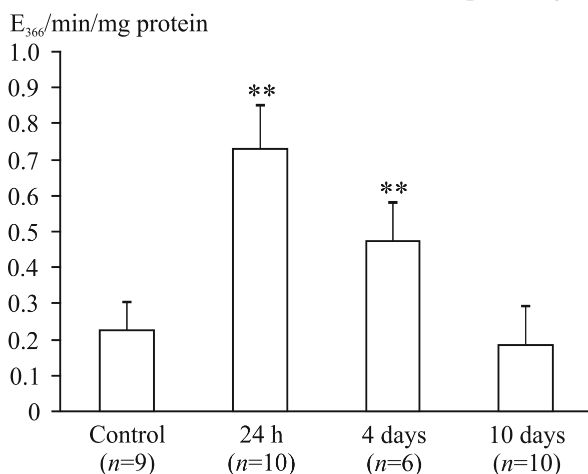

Fig. 1. Activity of catepsin D in spleen cells of mice with dyslipidemia, produced by repeated administration of P-407. Number of animals is shown in parentheses.**$p<0.001$ in comparison with the control.

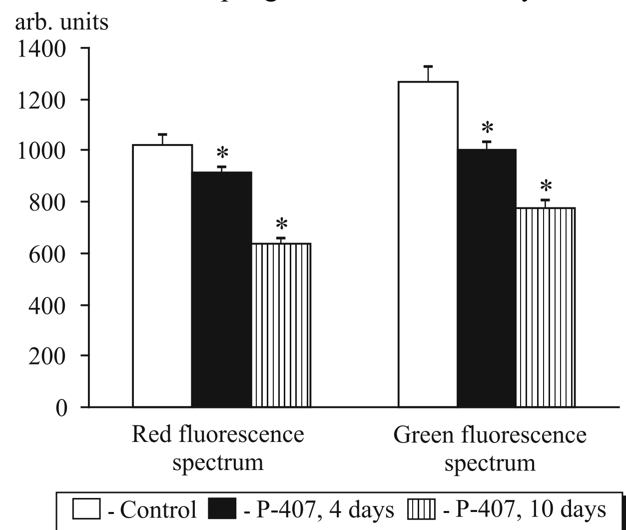

Fig. 2. Effects of P-407 administration to mice on accumulation of splenocytes to accumulate vital dye acridine orange. Ordinate: fluorescence intensity, arb. units. *p=0.0001 in comparison with the control.

Thus, the model of dyslipidemia in mice caused by repeated administration of relatively low doses of P-407 is close to the corresponding lipoprotein abnormalities in human atherosclerosis. A significant increase in the concentration of TC, atherogenic LDL, and TG (24 h) was revealed; normalization of the lipid profile was observed in 4 days (except TG) and 10 days. Probably, lipoproteins and P-407 accumulated in the liver and spleen macrophages modify the functions of these cells, which leads to an increase in spleen weight and lysosomal cathepsin D activity on the one hand, and impaired accumulation of vital dye acridine orange, on the other , which reflected decreased detoxifying function of lysosomes. Increased activity of spleen cathepsin D may be associated with lipid antigen presentation [11] and pleiotropic effect of cathepsin D [4,13,14]. The observed changes in the spleen cells are associated with dyslipidemia in the dynamics of recovery after repeated P-407 administration and demonstrate the association of hyperlipidemia with the state of peripheral immunity.

REFERENCES

1. V. M. Loginova, F. V. Tuzikov, N. A. Tuzikova, and T. A. Korolenko, *Bull Exp. Biol. Med.*, **155**, No. 2, 284-287 (2013).
2. A. V. Shurlygina, O. T. Kudaeva, O. P. Kolesnikova, *et al.*, *Bull Exp. Biol. Med.*, **156**, No. 1, 73-77 (2013).
3. F. Antunes, E. Enrique Cadenas, and U. T. Brunk, *Biochem. J.*, **356**, Pt 2, 549-555 (2001).
4. M. A. Bewley, H. M.Marriott, C. Tulone, *et al.*, *PLoS Pathog.*, **7**, No. 1, doi: 10.1371/journal.ppat.001001262 (2011).
5. M. K. Gould, X. L. Vu, T. Seebeck, and H. P. de Koning, *Anal. Biochem.*, **382,** No. 2, 87-93 (2008).
6. G. Dumortier, J. L. Grossiord, F. Agnely, and J. C. Chaumeil, *Pharm. Res.*, **23**, No. 12, 2709-2728 (2006).
7. T. P. Johnston, *J. Pharm. Pharmacol.*, **62**, No. 12, 1807-1812 (2010).
8. T. A. Korolenko, F. V. Tuzikov, M. S. Cherkanova, *et al.*, *Can. J. Physiol. Pharmacol.*, **90**, No. 2, 141-153 (2012).
9. T. A. Korolenko, F. V. Tuzikov, T. P. Johnston, *et al.*, *Can. J. Physiol. Pharmacol.*, **90**, No. 11, 1456-1468 (2012).
10. S. M. Moghimi, *Biochim. Biophys. Acta*, **1472**, Nos. 1-2, 399-403 (1999).
11. B. Nakken, T. Varga, I. Szatmari, *et al.*, *J. Immunol.*, **187**, No. 1, 240-247 (2011).
12. S. Sharyo, K. Kumagai, N. Yokota-Ikeda, *et al.*, *J. Pharmacol. Sci.*, **110**, No. 1, 47-54 (2009).
13. C. Tulone, Y. Uchiyama, M. Novelli, *et al.*, *BMC Immunol.*, **8**, 22 (2007).
14. A. Vashishta, S. S. Ohri, and V. Vetvicka, *Endocr. Metab. Immune Disord. Drug Targets*, **9**, No. 4, 385-391 (2009).
15. A. Warren, V. Benseler, V. C. Cogger, *et al.*, *Toxicol. Pathol.*, **39**, No. 2, 390-397 (2011).