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The level of markers of apoptosis and cell proliferation in the area of restenosis after lower extremity arterial reconstruction

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Abstract

Aim. To assess the number of markers of apoptosis and cell proliferation, as well as their relationships in the area of restenosis of arterial reconstructions.

Methods. The study included 14 patients with a diagnosis of "arteriosclerosis obliterans of the lower extremities. Post-thrombotic occlusion of femoropopliteal bypass". All patients were males with stage III disease according to the Fontaine classification modified by A.V. Pokrovsky. The average age of the patients was 65±3.4 years. The mean disease duration was 9±2.5 months after the initial intervention. Intraoperative material — distal anastomosis of femoropopliteal bypass — was taken from patients during arterial reconstructions. As a control, we used arterial wall samples obtained at organ procurement from postmortem donors without arteriosclerosis obliterans of the lower extremities. The number of samples is 8. The site of their collection is the popliteal artery. After sampling, they were crushed, and a homogenate was prepared, followed by the determination of the amount of p53, PDGF BB, Bcl2, and Bax proteins using the enzyme immunoassay. Statistical analysis was performed using the Statistica 10.0 software. Group differences were assessed by using the Mann–Whitney test. Correlation coefficients were determined using the Spearman test. Data are presented as medians and interquartile ranges.

Results. In tissue samples of restenosis, the amount of p53 protein was 0.07 units/mg and was significantly reduced compared with the control samples — 0.14 units/mg (p=0,015). The amount of platelet-derived growth factor PDGF BB was 0.17 ng/mg (p=0.05), Bcl2 — 1.61 ng/mg (p=0.008), Bax — 6.0 ng/mg (p=0.25) in the restenosis area and was increased in comparison with the control samples (0.04 ng/mg, 0.9 ng/mg, 4.4 ng/mg, respectively). A relationship between p53 and platelet-derived growth factor BB (r=–0.724, p=0.002), platelet-derived growth factor BB and Bcl2 (r=0.672, p=0.003) was revealed in samples from restenosis tissue obtained during arterial reconstructions.

Conclusion. The decreased apoptosis, expressed in a low level of p53 protein, with an increased Bax/Bcl-2 ratio is associated with an increase in the proliferative response of vascular wall cells in the area of restenosis of arterial reconstruction.

Keywords: Bcl2, Bax, p53, apoptosis, restenosis, PDGF BB.

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Background. At present, reconstructive and restorative interventions on the main arteries of the lower extremities have been increasing. The success of surgical treatment leads to the relief of lower limb ischemia and an improvement in the patient's quality of life. However, the development of restenosis of the intervention zone nullifies the success of operations and requires repeated reconstructions.

Neointimal hyperplasia (NI) is one of the leading causes of restenosis. The intimal proliferative response is part of the normal healing of the vascular wall following surgical trauma. However, in several cases, uncontrolled NI hyperplasia develops, but its prevention and control remain unaddressed [1].

Recently, several studies of the pathogenesis of this complication have shown that the apopto-

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sis system can play an important role in the development of NI hyperplasia [2]. Apoptosis is defined as genetically programmed cell death that plays a key role in the regulation of the cellular composition of various tissues, including the arterial wall, in both normal conditions and atherosclerotic lesions [3, 4].

Modern diagnostic methods, such as light microscopy and flow cytometry, make it possible to determine morphological signs of apoptosis — decreased cell size, wrinkling of the cytoplasmic membrane, nucleus condensation, rupture of nuclear deoxyribonucleic acid (DNA) filaments, etc. A more informative method is to determine biochemical markers of apoptosis, giving practical points of the application of therapeutic strategies in the treatment of patients with obliterating atherosclerosis of the arteries of the lower extremities (OAANK) [5].

In the literature databases of PubMed, eLibrary, Google Scholar, and Medline, no studies have investigated laboratory markers of apoptosis in the restenosis zone in patients with OAANC.

Proteins of the Bcl2 and p53 families are the main markers involved in the regulation of the apoptosis system in the cells of the vascular wall [6].

p53 is a stress-dependent protein that is activated by various stimuli, such as DNA damage, hypoxia, and oxidative stress in atherosclerosis. After activation, it inhibits the phase change of the cell cycle, thereby maintaining a stable cell composition. It participates in the regulation of apoptosis either through proteins of the Bcl2 family (inducing the proapoptotic protein Bax upon inhibition of the antiapoptotic protein Bax) or through the receptor pathway (inducing the interaction of the Fas receptor with the Fas ligand with subsequent activation of caspases) [7].

Cao et al. (2017) showed accelerated development of atherosclerotic lesions in mice lacking the p53 protein gene, and smooth muscle cells (SMCs) have an increased proliferation rate against the background of their insignificant death [8].

Bcl2 family of proteins is another active participant in the apoptosis system. Its main representatives are the antiapoptotic protein Bcl2 and the proapoptotic protein Bax. The Bcl2/Bax ratio in the mitochondrial membrane determines the fate of the cell; thus, it is called the proapoptotic index. These proteins were found in cells of atherosclerotic plaques (mainly SMCs and macrophages) at different localizations of the lesion [9]. Several scientists showed that two opposite cellular processes, i.e., proliferation and apoptosis, exist together in the restenosis zone of the rat carotid artery after balloon angioplasty [10].

The role of cell proliferation and migration in the formation of NI hyperplasia in the restenosis zone has been proven by experimental and clinical studies. Platelet-derived growth factor BB (PDGF BB) is one of the key representatives of cell proliferation and migration, which is involved in the formation of NI hyperplasia. In response to surgical trauma, it is activated in the first hours and induces the migration of SMCs from the media to the intima. The use of PDGF BB inhibitors in experimental models led to a decrease in NI formation. However, the relationship and correlation of PDGF BB with apoptosis markers in the development of NI hyperplasia have not been sufficiently evaluated, and available data are contradictory [11].

Based on the foregoing data, this study aimed to assess some markers of apoptosis and cell proliferation, as well as their relationships, in the restenosis zone of arterial reconstructions.

Materials and methods. The cohort study included 14 patients from 2019 to 2020 with a diagnosis of OAANK. Post-thrombotic occlusion of synthetic femoropopliteal shunts were placed above the knee joint gap. All patients were male with stage III disease according to the classification by Pokrovsky and Fontaine. The average age of the patients was 65 ± 3.4 years, and the average disease duration after the initial reconstructive intervention was 9 ± 2.5 months.

The study protocol was approved by the local ethics committee of the Ryazan State Medical University named after I.I. I.P. Pavlova (No. 7, dated March 03, 2020).

After an additional examination with ultrasound duplex scanning and angiography of the arteries of the lower extremities), the patients underwent repeated arterial reconstructions at the Regional Clinical Hospital in Ryazan. Intraoperatively, a sample was taken, which was a distal anastomosis of a synthetic femoral–popliteal shunt (an early shunt artery, a section of the prosthesis itself with a neointimal lining).

As a control, we used arterial wall samples obtained during organ explantation from postmortem donors without OAANC (according to ultrasound duplex scanning of the arteries of the lower extremities). Eight samples were collected from the popliteal artery. All donors were male, with an average age of 61 ± 4.4 years. No differences in age were found between the study group and control group ($p = 0.143$).

The samples were ground, and a homogenate was prepared using a lysis buffer (Thermo Fisher Scientific, USA) and a DIAX 900 rotary high-speed homogenizer (Heidolph, Germany) (6G nozzle), at a speed of 24,000 rpm for 60 s and temperature of

Table 1. Markers of apoptosis and cell proliferation in patients with restenosis of the reconstruction zone

Parameters, Me [Q_1–Q_3]	P53, U/mg	PDGF BB, ng/mg	Bcl2, ng/mg	Bax, ng/mg	Bcl2/Bax
Samples with restenosis of the arterial reconstruction zone	0.07 [0.06; 0.08]	0.17 [0.09; 0.34]	1.61 [1.21; 1.8]	6.0 [5.1; 7.2]	0.28
Samples with normal arterial wall	0.14 [0.09; 0.24]	0.04 [0.02; 0.09]	0.9 [0.77; 1.26]	4.4 [3.2; 6.7]	0.20
p	0.015*	0.05*	0.008*	0.25	0.473

Note: * significant difference ($p < 0.05$); Me, median; Q_1–Q_3, lower and upper quartiles; PDGF BB, platelet-derived growth factor BB.

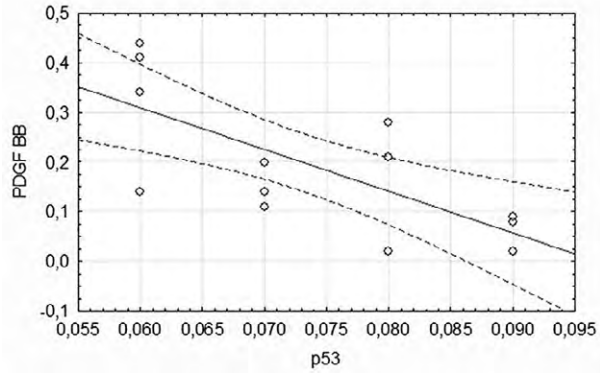

Fig. 1. Inverse correlation between p53 and platelet-derived growth factor BB in patients with restenosis of the reconstruction zone

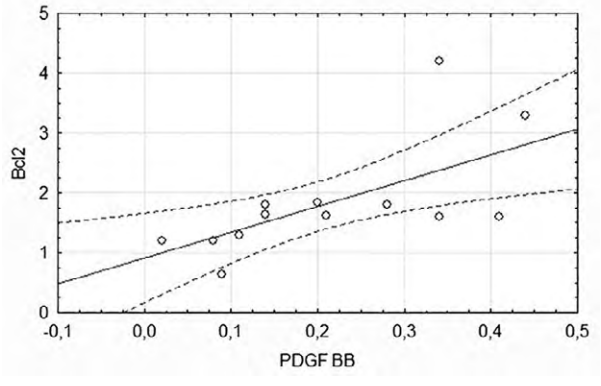

Fig. 2. Direct correlation between platelet-derived growth factor BB and Bcl2 in patients with restenosis of the reconstruction zone

+2°C. The resulting homogenate was centrifuged at 1000 g for 10 min (temperature + 2°C). In the resulting supernatant, the amounts of Bcl-2, p53, and PDGF BB were determined using a commercial enzyme-linked immunosorbent assay kit from Invitrogen (MA, USA), and the level of Bcl2-associated X protein (Bax) was determined using a kit from Cloud-Clone Corp. (China). The obtained values were recalculated for the protein content, which was estimated by the Bradford method using the Coomassie Plus (Bradford) AssayKit (Thermo Fisher Scientific, MA, USA).

Statistical analyses of the data obtained were performed using Statistica 10.0 statistical software package. The distribution of indicators was evaluated using the Shapiro–Wilk test ($p > 0.05$). Owing to the deviation from the normal distribution of the data, nonparametric tests were used for further analysis. Group differences were assessed using the Mann–Whitney test. Correlation coefficients were determined using Spearman's test. Data are presented as median and interquartile range, i.e., Me (Q1–Q3). The accepted level of statistical significance was $p < 0.05$.

Results. The amount of p53 in the samples from the restenosis zone was two times lower than the values in the normal arterial wall ($p = 0.015$). The amount of PDGF BB in the samples from the restenosis zone exceeded by 23.5% its amount in the control samples ($p = 0.05$). In the restenosis zone, the amount of antiapoptotic protein Bax was increased by 59% ($p = 0.008$), the proapoptotic protein Bax by 73% ($p = 0.25$), and the Bcl2/Bax ratio by 71% ($p = 0.473$) compared with their amounts in the control samples (Table 1).

Correlation analysis revealed a relationship between p53 and PDGF BB ($r = 0.724$, $p = 0.002$), as well as between PDGF BB and Bcl2 ($r = 0.672$, $p = 0.003$) in samples with restenosis of the intervention zone (Figs. 1, 2). In control samples, a direct relationship was found between PDGF BB and Bax ($r = 0.754$, $p = 0.031$).

Discussion. The results of the study showed that cell apoptosis and cell proliferation are balanced in the normal arterial wall. The correlation between PDGF BB and the proapoptotic protein Bax proves that these two processes are interrelated and work in balance with each other. Normally, in response to cell death in the vascular wall, compensatory replacement occurs due to an increase in the proliferation and migration of neighboring cells because of the growth of new ones under the influence of mitogenic signals produced by apoptotic cells [12].

In this study, we obtained a completely different picture in the samples with restenosis of the intervention zone. Increased PDGF BB level indicates a high proliferative activity of cells in the restenosis zone. Cell proliferation, formation, and remo-

deling of the extracellular matrix are well-known mechanisms for the formation of restenotic lesions.

Moreover, low amounts of p53 could not induce the activation of apoptosis through Bax; however, given its controlling function in the regulation of the cell cycle, it promoted cell proliferation, enhancing the mitogenic effect of PDGF BB, which was confirmed by the correlation analysis. p53 is mainly involved in stopping the cell cycle, regulation of the apoptosis system, and suppression of cell proliferation and migration, which have been proven in various works. Thus, increased expression of the p53 protein gene leads to a decrease in intimal thickening by approximately 80% in the postoperative period [13]. This finding is caused by the increased amount of p53, which leads to a decrease in the incorporation of thymidine into proliferating SMCs, stimulated by PDGF BB. Subsequently, the phase of the change of the phenotype to the synthetic one ends faster in these cells; therefore, they proliferate less and migrate to the forming NI.

George demonstrated that overexpression of p53 in animals promotes apoptosis activation upon inhibition of SMC migration, which leads to a decrease in NI thickness after autovenous shunting [14]. Bauriedel et al. used the terminal deoxynucleotidyl transferase dUTP nick end labeling method and showed that the restenotic region contained fewer apoptotic cells than the atherosclerotic plaque [15]. Interestingly, Scott used brachytherapy for the treatment of restenosis, which led to the activation of p53 and the induction of apoptosis in restenotic SMCs. These cells were found to be more sensitive to p53-induced apoptosis than intact cells of the vascular wall [16].

Interestingly, in these samples, we found a significant increase in the amount of the antiapoptotic protein Bcl2. In previous studies, cells in the area of NI hyperplasia are less sensitive to apoptosis than media cells. However, the mechanism of this process has not been fully determined [17].

We can assume that the increased level of Bcl2 protects the proliferating NI cells from death, increasing their life (direct correlation between Bcl2 and PDGF BB). Such an increase in the amount of Bcl2 may be due to a change in the SMC phenotype from being contractile to being synthetic following surgical trauma. Conversely, p53 triggers apoptosis through the Bcl2 family of proteins (increasing the Bax/Bcl2 ratio), and its decreased level could contribute to an increase in the Bcl2 amount.

In restenosis samples, the amount of Bax was increased, but was not significant; thus, it could not balance such an increased proliferative response, which may have influenced the development of this complication.

The results of study are limited because only the main markers of the mitochondrial pathway of apoptosis (proteins Bcl2 and Bax) were identified. In our opinion, it is also necessary to evaluate the state of the receptor pathway of apoptosis, i.e., the "Fas receptor–Fas ligand" system and its relationship with Bcl2, Bax, p53, and PDGF BB. Further investigation for indicators leading to the development of restenosis of the reconstruction zone will make it possible to find new strategies to prevent this complication.

CONCLUSIONS

1. Decreased apoptotic activity, expressed in low values of p53 (0.07 U/mg) due to an increased Bcl2/Bax ratio (0.28), leads to an increase in the proliferative response of vascular wall cells in the restenosis zone of arterial reconstruction.

2. Correlation analysis revealed a relationship between p53 and PDGF BB ($r = -0.724$, $p = 0.002$) and between PDGF BB and Bcl2 ($r = 0.672$, $p = 0.003$) in samples with restenosis of the reconstruction zone.

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REFERENCES

1. Zhu Z.R., He Q., Wu W.B., Chang G., Yao C., Zhao Y., Wang M., Wang S.M. MiR-140-3p is involved in in-stent restenosis by targeting C-Myb and BCL-2 in peripheral artery disease. *J. Atheroscler. Thromb.* 2018; 25 (11): 1168–1181. DOI: 10.5551/jat.44024.

2. Zhu H., Zhang Y. Life and death partners in post-PCI restenosis: Apoptosis, autophagy, and the cross-talk between them. *Curr. Drug Targets.* 2018; 19 (9): 1003–1008. DOI: 10.2174/1389450117666160625072521.

3. Kalinin R.E., Suchkov I.A., Klimentova E.A., Egorov A.A. To the question of the role of apoptosis in the development of atherosclerosis and restenosis of the reconstruction zone. *Novosti khirurgii*. 2020; 28 (4): 418–427. (In Russ.) DOI: 10.18484/2305-0047.2020.4.418.

4. Klimentova E.A., Suchkov I.A., Egorov A.A., Kalinin R.E. Apoptosis and cell proliferation markers in inflammatory-fibroproliferative diseases of the vessel wall (review). *Sovremennye tekhnologii v meditsine.* 2020; 12 (4): 119–128. (In Russ.) DOI: 10.17691/stm2020.12.4.13.

5. Banfalvi G. Methods to detect apoptotic cell death. *Apoptosis.* 2017; 22 (2): 306–323. DOI: 10.1007/s10495-016-1333-3.

6. Gross A., Katz S.G. Non-apoptotic functions of BCL-2 family proteins. *Cell Death Differ.* 2017; 24 (8):

1348–1358. DOI: 10.1038/cdd.2017.22.

7. Kolovou V., Tsipis A., Mihas C., Katsiki N., Vartela V., Koutelou M., Manolopoulou D., Leondiadis E., Iakovou I., Mavrogieni S., Kolovou G. Tumor protein p53 (TP53) gene and left main coronary artery disease. *Angiology.* 2018; 69 (8): 730–735. DOI: 10.1177/0003319718760075.

8. Cao R.Y., Eves R., Jia L., Funk C.D., Jia Z., Mak A.S. Effects of p53-knockout in vascular smooth muscle cells on atherosclerosis in mice. *PLoS One.* 2017; 12 (3): e0175061. DOI: 10.1371/journal.pone.0175061.

9. Kale J., Osterlund E.J., Andrews D.W. BCL-2 family proteins: changing partners in the dance towards death. *Cell Death Differ.* 2018; 25 (1): 65–80. DOI: 10.1038/cdd.2017.186.

10. Igase M., Okura T., Kitami Y., Hiwada K. Apoptosis and Bcl-xs in the intimal thickening of balloon-injured carotid arteries. *Clin. Sci. (Lond.).* 1999; 96 (6): 605–612. PMID: 10334966.

11. Chen S., Dong S., Li Z., Guo X., Zhang N., Yu B., Sun Y. Atorvastatin calcium inhibits PDGF-ββ-induced proliferation and migration of VSMCs through the G0/G1 cell cycle arrest and suppression of activated PDGFRβ-PI3K-Akt signaling cascade. *Cell Physiol. Biochem.* 2017; 44 (1): 215–228. DOI: 10.1159/000484648.

12. Aravani D., Foote K., Figg N., Finigan A., Uryga A., Clarke M., Bennett M. Cytokine regulation of apoptosis-induced apoptosis and apoptosis-induced cell proliferation in vascular smooth muscle cells. *Apoptosis.* 2020; 25 (9–10): 648–662. DOI: 10.1007/s10495-020-01622-4.

13. Yonemitsu Y., Kaneda Y., Tanaka S., Nakashima Y., Komori K., Sugimachi K., Sueishi K. Transfer of wild-type p53 gene effectively inhibits vascular smooth muscle cell proliferation *in vitro* and *in vivo*. *Circ. Res.* 1998; 82 (2): 147–156. DOI: 10.1161/01.res.82.2.147.

14. George S.J., Angelini G.D., Capogrossi M.C., Baker A.H. Wild-type p53 gene transfer inhibits neointima formation in human saphenous vein by modulation of smooth muscle cell migration and induction of apoptosis. *Gene Ther.* 2001; 8 (9): 668–676. DOI: 10.1038/sj.gt.3301431.

15. Bauriedel G., Hutter R., Schluckebier S., Welsch U., Prescott M.F., Kandolf R., Lüderitz B. Decreased apoptosis as a pathogenic factor in intimal hyperplasia of human arteriosclerosis lesions. *Z. Kardiol.* 1997; 86 (8): 572–580. DOI: 10.1007/s003920050096.

16. Scott S., O'Sullivan M., Hafizi S., Shapiro M., Bennett M.R. Human vascular smooth muscle cells from restenosis or in-stent stenosis sites demonstrate enhanced responses to p53: implications for brachytherapy and drug treatment for restenosis. *Circ. Res.* 2002; 90 (4): 398–404. DOI: 10.1161/hh0402.10590.

17. Walsh K., Smith R.C., Kim H.S. Vascular cell apoptosis in remodeling, restenosis, and plaque rupture. *Circ. Res.* 2000; 87 (3): 184–188. DOI: 10.1161/01.res.87.3.184.