Results of long-term permeability of small-diameter vascular prostheses modified by pro-angiogenic factors and athrombogenic drug coating (model of large laboratory animals)

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Abstract. Developed for bypass surgery, tissue-engineered vascular grafts should remain passable after implantation and have high rate of endothelialization to ensure long-term patency, athrombogenicity and biocompatibility. This study was aimed at assessing the long-term patency of polyhydroxybutyrate/valerate (PHBV)/polycaprolactone (PCL) vascular grafts modified by pro-angiogenic factors and athrombogenic drug coating in a large laboratory animal model.

1. Introduction
Tissue-engineered small diameter vascular grafts are the alternative to the deficiency of autologous blood vessels in the surgical treatment of cardiovascular diseases. Consisting of biocompatible and biodegradable polymers, these functionally active grafts have been developed all over the world [1]. However, tissue-engineered vascular grafts must have a high patency, athrombogenicity and biocompatibility [2]. One of the ways to increase biocompatibility in polymer grafts is to incorporate growth factors (GFmix) into the tubular graft sheath and additionally modify the surface by antiplatelet agents and anticoagulants [1,3].

2. Materials and methods

2.1. Fabrication of tissue-engineered vascular grafts
Biodegradable vascular grafts (4 mm diameter) were prepared by electrospinning (Nanon-01A, MECC, Japan) from 14% (poly(ε-caprolactone) PCL and 10% (polyhydroxybutyrate/valerate) PHBV solution dissolved in chloroform, with the incorporation of bioactive molecules (GFmix). Vascular endothelial growth factor (VEGF; Sigma-Aldrich, USA) was incorporated into the inner third of the graft wall. The basic fibroblast growth factor (bFGF; Sigma-Aldrich, USA) and stromal cell–derived factor-1α (SDF-1α; Sigma-Aldrich, USA) were incorporated into the outer two thirds of the graft wall.

2.2. Surface modification of the polymer grafts by anticoagulants and antiplatelet agents
Using authors’ original method [4], the inner surface of the graft was additionally modified by antiplatelet agents and anticoagulants to increase the thrombosis resistance. The antiplatelet and
anticoagulant agents - iloprost and unfractionated heparin, respectively, were used as antithrombotic drugs. Using the method of radiation-induced polymerization, polyvinylpyrrolidone (PVP) hydrogel coating was formed on the inner surface of the sheath to bind heparin and iloprost. Firstly, the graft was submerged in a PVP solution in ethanol at a concentration of 1-25% for 30 minutes. Then the prosthesis was removed from the solution, air-dried for 24 hours, placed in argon-filled glass vials and irradiated at 25-50 kGy. To bind heparin and iloprost to the PVP hydrogels, vascular prostheses were kept in a modifying solution of unfractionated heparin and iloprost in a glycine buffer solution for 30 minutes. The grafts were then air-dried under sterile conditions.

2.3. In Vivo Implantation

PHBV/PCL/GFmix\textsubscript{Hep/Ilo} prostheses of 4 mm diameter and 40 mm length were implanted into the carotid artery of a sheep, according to the scheme: 1 animal – 1 prosthesis (n=8); resection of a section of the carotid artery of 40 mm length for the implantation of the graft using the “end-to-end” method. The implantation period is 18 months. The comparison group - Gore-Tex\textsuperscript{®} vascular prostheses (Gore-Tex, USA) (n=5) were implanted into sheep carotid artery for 6 months. Ultrasound dopplerography was performed on the M7 Premium device (Mindray, China) to assess the patency of the implanted grafts: at days 1 and 5, months 1, 3, 6, 9, 12, 15 and 18 following surgery – for passable vascular prostheses; at days 1 and 5 – for thrombosed prostheses.

2.4. Histological examination

PHBV/PCL/GFmix\textsubscript{Hep/Ilo} grafts were explanted after 1.5 years of implantation, whereas Gore-Tex grafts were explanted after 6 months of implantation. To determine the presence of calcium, histological examination of explanted samples stained with hematoxylin-eosin, Van Gieson, orcein, alizarin red S and DAPI was performed. The explanted samples were fixed in formalin for 24 hours, rinsed with running tap water and dehydrated in IsoPrep (BioVitrum, Russia). Then they were soaked 3x in paraffin at 56 °C for 60 minutes. Impregnated samples were immersed in HISTOMIX\textsuperscript{®} paraffin (BioVitrum, Russia). 8 μm sections were cut using the HM 325 microtome (Thermo Scientific, USA), placed in a thermostat and dried overnight at 37 °C. After drying, they were dewaxed 3x in o-xylene for 1-2 minutes and dehydrated 3x in 96% ethanol for 1-2 minutes. Following that, the dewaxed sections were stained in accordance with the study protocol. The sections were evaluated by light and fluorescence microscopy (AXIO Imager A1 microscope with 50x, 100x and 200x magnification, Carl Zeiss, Germany).

2.5. Immunofluorescence examination

The explanted grafts were frozen at -120 °C and fixed in a Tissue-Tek embedding medium (Sakura, Japan). 8 μm sections were cut using a CryoStar NX50 cryostat (Thermo Scientific, USA). Sections were stained with antibodies to CD31, vWF, α-actin, collagen I, III, IV type (Abcam, England), cell nuclei were contrasted with DAPI (Sigma, USA). Control samples were prepared similarly to experimental ones, but 1% BSA was used instead of primary antibodies. Slides were mounted onto a labeled glass slide with ProLong (Life Technologies) and analyzed using a laser scanning microscope LSM 700 (Zeiss, Germany).

3. Results and discussion

3.1. Results of histological assessment

A day after implantation, 100% of Gore-Tex prostheses and 37.5% of PHBV/PCL/GFmix\textsubscript{Hep/Ilo} grafts were thrombosed. After 10 months of implantation, another PHBV/PCL/GFmix\textsubscript{Hep/Ilo} graft was thrombosed. Thus, the patency of this group of prostheses after 1.5 years of implantation was 50%. Aneurysms were found in all passable explanted PHBV/PCL/GFmix\textsubscript{Hep/Ilo} grafts (figure 1).
Figure 1. Image (a) and patency (b) of a PHBV/PCL/GFmix\textsuperscript{Hep\textsubscript{IIo}} graft after 1.5 years of implantation.

A histological study of explanted samples showed that the biodegradable sheath of PHBV/PCL/GFmix\textsuperscript{Hep\textsubscript{IIo}} thrombosed grafts was almost completely resorbed (figure 2). Formation of a newly formed three-layer vessel similar in structure to the native carotid artery of a sheep was revealed in the place of the biodegradable tubular sheaths. A layer of endothelial cells was visualized on the inner side of the neointima of the newly formed vessel. Media is represented by cells similar in morphology to smooth muscle cells. The smooth muscle cells were followed by a layer of connective tissue and adventitia, which contained a large number of newly formed vessels, lymphatic follicles and a small amount of perivascular adipose tissue. In addition, the adventitia structure contained a small number of fibroblast-like cells and foreign body cells. However, unlike the native vessel, the newly formed vascular tissue had no elastic fibers and a clear elongation of the cytoplasm of smooth muscle cells, which probably caused aneurysmal stretching of the newly formed vascular tissue due to pulsating blood flow. Additionally, a small focus of calcium was found under the neointima in the wall of one of the four passable grafts.

| Hematoxylin-eosin | Van Gieson | Orsein | Alizarin red S |
|-------------------|------------|--------|---------------|
| Neointima | Media | Adventitia |

Figure 2. Results of histological examination of PHBV/PCL/GFmix\textsuperscript{Hep\textsubscript{IIo}} grafts after 1.5 years of implantation, 100x magnification.

After 6 months of implantation, obturating recanalized thrombus and foci of crystalline calcium were detected in the lumen of Gore-Tex prostheses both in the wall of the prosthesis and in adjacent connective tissue capsule (figure 3).
Figure 3. Results of histological examination of Gore-Tex grafts explanted 6 months following surgery. The arrow indicates the place with crystalline calcium in the capsule of the prosthesis, 100 x magnifications.

3.2. Results of the immunofluorescence study
The presence of endothelium containing signs of endothelial-to-mesenchymal transition (simultaneous expression of CD31 and α-actin, vWF) was noted in passable PHBV/PCL/GFmix<sub>Hep/Ilo</sub> grafts. The basal membrane contained type I and type IV collagen. Type III collagen is also found in the graft wall. The lumen of the Gore-Tex vessels was filled with a blood clot. The formation of newly formed tissue in the prostheses of this group was absent (figure 4).

|                | a                      | b                      | c                      |
|----------------|------------------------|------------------------|------------------------|
| CD 31          |                        |                        |                        |
| CD 34          |                        |                        |                        |
| DAPI           |                        |                        |                        |
| Collagen I     |                        |                        |                        |
| Collagen IV    |                        |                        |                        |
| DAPI           |                        |                        |                        |
| vWF            |                        |                        |                        |
| DAPI           |                        |                        |                        |
| Collagen III   |                        |                        |                        |
| DAPI           |                        |                        |                        |

Figure 4. Comparison of the results of an immunofluorescence study of PHBV/PCL/GFmix<sub>Hep/Ilo</sub> prostheses and Gore-Tex prostheses. a - Gore-Tex; b – passable PHBV/PCL/GFmix<sub>Hep/Ilo</sub> prostheses; c – thrombosed PHBV/PCL/GFmix<sub>Hep/Ilo</sub> prostheses, 200x magnification.
4. Conclusion
The results of the study demonstrated high biocompatibility of PHBV/PCL/GFmix\textsuperscript{Hep/Ilo} vascular prostheses compared to Gore-Tex prostheses during long-term implantation. The PHBV/PCL/GFmix\textsuperscript{Hep/Ilo} biodegradable scaffolds tested in this study have shown the ability to assist with the formation of vascular tissue, but they require sheath reinforcement in order to prevent thrombosis. It is also necessary to further increase non-thrombogenous properties of the inner surface of the modified prostheses.

Acknowledgements
The work was performed within the framework of the program world-class Research and Education center “Kuzbass”

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