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Evaluation of the humoral and cellular immune responses after implantation of a PTFE vascular prosthesis*

Ocena immunologicznej odpowiedzi humoralnej i komórkowej po zabiegach wszczepienia protezy naczyniowej z PTFE

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: The experiment was designed in order to determine the immunological processes that occur during the healing in synthetic vascular grafts, especially to establish the differences in the location of the complement system proteins between the proximal and distal anastomosis and the differences in the arrangement of inflammatory cells in those anastomoses. The understanding of those processes will provide a true basis for determining risk factors for complications after arterial repair procedures.

Material/Methods: The experiment was carried out on 16 dogs that underwent implantation of unilateral aorto-femoral bypass with expanded polytetrafluoroethylene (ePTFE). After 6 months all animals were euthanized to dissect the vascular grafts. Immunohistochemical assays and electron microscopic examinations were performed.

Results: Immunohistochemical findings in the structure of neointima between anastomoses of vascular prostheses demonstrated significant differences between humoral and cellular responses. The area of proximal anastomosis revealed the presence of fibroblasts, but no macrophages were detected. The histological structure of the proximal anastomosis indicates that inflammatory processes were ended during the prosthesis healing. The immunological response obtained in the distal anastomosis corresponded to the chronic inflammatory reaction with the presence of macrophages, myofibroblasts and deposits of complement C3.

Discussion: The identification of differences in the presence of macrophages and myofibroblasts and the presence of the C3 component between the anastomoses is the original achievement of the present study. In the available literature, no such significant differences have been shown so far in the humoral and cellular immune response caused by the presence of an artificial vessel in the arterial system.

Key words: **vascular prosthesis • PTFE • neointima • complement • myofibroblasts • collagen • macrophages**

* The study was conducted with the consent of the Ethics Committee for Scientific Research.

Streszczenie

Wprowadzenie: Eksperyment został zaprojektowany w celu określenia procesów odpowiedzi humoralnej i komórkowej w przebiegu wgajania się syntetycznej protezy naczyniowej. Celem głównym było określenie różnic w lokalizacji białek układu dopełniacza między proksymalnym i dystalnym zespoleniem oraz różnic w rozmieszczeniu komórek odpowiedzi zapalnej w tych zespoleniach. Zrozumienie tych procesów będzie podstawą określania czynników ryzyka powikłań po przeprowadzanych procedurach rekonstrukcji naczyniowych.

Materiał/Metody: Badania zostały przeprowadzone na 16 psach, które poddane zostały zabiegowi implantacji jednostronnego przęsła aortalno-udowego z ekspandowanego politetrafluorotylenu (ePTFE). Po upływie sześciu miesięcy wszystkie zwierzęta usypiano i sekcjonowano, pobierając przęsło wraz marginesem tkanek Wykonywano badania immunohistochemiczne określające złogi czynnika C3 układu dopełniacza oraz badania w mikroskopie elektronowym z okolic zespolenia proksymalnego i dystalnego protezy z układem tętniczym.

Wyniki: Wyniki badań immunohistochemicznych neointimy między zespoleniami protezy naczyniowej wykazały istotne różnice między odpowiedzią humoralną i komórkową. W okolicy zespolenia proksymalnego wykazano obecność fibroblastów, nie obserwowano natomiast makrofagów. Struktura histologiczna zespolenia proksymalnego świadczy o tym, że procesy zapalne uległy wygaszeniu w trakcie wgajania się protezy. Uzyskany obraz zespolenia dystalnego odpowiadał z kolei przewlekłej reakcji zapalnej z obecnością makrofagów, miofibroblastów oraz istotnie statystycznie zwiększonej ilości złogów czynnika C3 układu dopełniacza.

Dyskusja: Oryginalnym wynikiem przedstawionego badania jest identyfikacja różnic w obecności makrofagów, miofibroblastów oraz składnika C3 układu dopełniacza między zespoleniami syntetycznej protezy naczyniowej z układem tętniczym. W dostępnej literaturze nie znaleziono prac, które określałyby tak istotne różnice w immunologicznej odpowiedzi humoralnej i komórkowej wywołanej obecnością syntetycznego naczynia w układzie tętniczym.

Słowa kluczowe: **proteza naczyniowa • neointima • dopełniacz • miofibroblasty • kolagen • makrofagi**

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Introduction

In order to reduce the incidence of complications that limit the durability of a synthetic vascular graft it is necessary to investigate the humoral and cellular inflammatory response at the site of vascular graft implantation [7,8,11,22].

Immediately after implantation of a vascular prosthesis, a mural thrombus is formed that is attached to the inner prosthetic wall. The release of thrombin/factor II/and mural aggregation of platelets cause activation of the complement system. For example, P-selectin, a platelet alpha granule membrane protein that contains a short consensus repeat domain common to many complement binding proteins, has been identified as an activator of the alternative complement cascade on platelets. Platelets also express binding sites for classical complement components, most notably for C3. C3 interactions with platelets trigger a variety of cellular and biochemical responses that may contribute to inflammation and thrombosis [14].

Complement activation involves a series of cascade-type enzymatic reactions. It means that every activated component activates another one [10,14]. In the course of activation, two very important enzymes are formed, C3 convertase and C5 convertase, which enhance the complement effects very strongly. However, regardless of the activation mechanism, the final stage is the formation of a membrane attack complex (MAC) which consists of C5b, C6, C7, C8 and polymeric C9, and the release of chemotactic factors for neutrophils and monocytes [1,10,14]. The inflammatory cell-mediated reorganisation of mural thrombus begins with neutrophil and monocyte adhesion and aggregation. Next, macrophages, fibroblasts, and myofibroblasts contribute to the formation of neointima, which is a very specific poorly cellular layer of extracellular matrix [4,13,26].

Immunohistochemical assays of complement system cascade proteins in vascular anastomoses allow one to evaluate the mechanisms of humoral immune response to the implantation of a synthetic vascular graft in the arterial system [1,10,13,14,22].

Table 1. The expression of C3 factor in proximal and distal anastomosis

Feature	Factor	Symbol	Mean value	Mediana	SD	Quarterly interval	Student's t-test
Proximal anastomosis	C3	PC3	188.250	189.250	9.124	4.760	10.743
Distal anastomosis	C3	DC3	219.654	218.500	6.347	5.450	10.743

P<0.05.

Based on electron microscope analysis, it is possible to evaluate the activity and contribution of the inflammatory cells in the repair processes, as well as in the degenerative processes which occur in the vascular prosthesis wall at a site of reorganization of a mural thrombus into the inner layer, i.e. neointima [2,9,18].

The obtained data will allow identification of the mechanisms of humoral and cellular immune response after implantation. The explanation of processes occurring in the histological structure of vascular anastomoses would provide a true basis for determining risk factors for complications after arterial repair procedures [7,11].

The aim of this study was to compare the presence of the complement and the inflammatory cells in the neointima after implantation of a vascular graft. The experimental model was designed based on data from the literature [4,24,26].

The following questions were formulated:
1. Is there any difference in the location of the complement system proteins between the proximal and distal anastomosis of a synthetic vascular graft?
2. Is there any difference in the arrangement of inflammatory cells in the proximal and distal anastomosis of a synthetic vascular graft?
3. Are there any differences in the histological structure of the neointima between the proximal and distal anastomosis?

Material and Methods

After obtaining approval from the Ethics Committee, a group of 16 mongrel dogs, weighing 20–26 kg and aged from 2 to 4 years, was selected and qualified as the research material. Dogs were operated on in general intratracheal anaesthesia, after a transperitoneal surgical approach in the right groin area. The animals were implanted with a unilateral aorto-femoral bypass, 6 mm 5-tetrafluoroethane (ePTFE) prosthesis. The length of the prosthesis varied from 8 cm to 12 cm. After 6 months all animals were euthanized in order to dissect the implanted vascular grafts, together with the cell margin.

Immunohistological examinations of complement component 3

The samples for immunohistological study were paraffinized scraps, 4 μm thick. They were placed on silanized glass plates (DAKO® cat. number S 3003), deparaffinized in xylene and transferred through a graded series of alcohols (decreasing concentration until water).

Tissue antigens, fixed in paraffin, were detected in Target Retrieval Solution (DAKO® cat. number S 1700) by heating in a water bath (96°C, 30 min.). Endogenous peroxidase was blocked with 3% H_2O_2 (10 min.), then the scraps were covered with the primary antibodies (Polyclonal Rabbit Anti-Human C3c Complement, code number 0062, DAKO dilution 1/200), incubated for 30 min. at room temperature, then washed in TBS and covered with biotinylated antibody (LSAB® + Kit, DAKO K 0679) for 15 min. After washing in TBS, scraps were incubated for 15 min. with streptavidin-peroxidase complex (LSAB® + Kit, DAKO K 0679).

Immunohistochemical reaction was triggered by a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB +, Liquid K 3486 DAKO), then the scraps were washed in running water and dehydrated through alcohol series. The material was x-rayed in xylene and enclosed in balsam.

Electron microscope examination

The samples were collected from both the proximal anastomosis (aortas) and distal anastomosis (common femoral arteries), with the ePTFE prosthesis. The obtained segments were fixed in 2.5% glutaraldehyde solution in 0.1 M. cacodyl buffer at 4°C for 12 hours and then in 1% OsO4 in cacodyl buffer at pH 7.4 for 1 hour. The slices were embedded in Epson-812. The semi-thin sections were stained with toluidine blue solution and preliminarily examined under a light microscope in order to select the most convenient trimming area, i.e. a vessel and graft vessel intersection. The ultra thin sections were stained with lead citrate and uranyl acetate and then examined under the JEM100B electron microscope.

All slices were assessed by the histology specialist from the Electron Microscope Laboratory at the Institute of Histology at the Wroclaw Medical University.

The tool used to analyse the results was the Student's t-test.

Results

All animals survived the surgical procedure and the full six-month period of clinical follow-up. In all cases the clinical course was uncomplicated.

Immunohistochemical analysis

The expression of C3 factor in the proximal and distal anastomosis was analysed with the computer scanning system Multi Scan 5.10. Histograms were expressed in numerical data (Table 1).

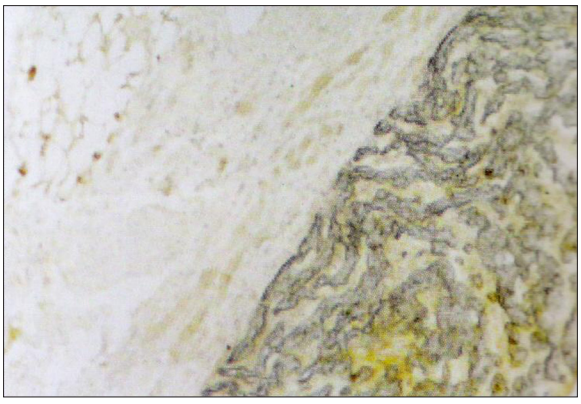

Fig. 1. C3 deposits in proximal anastomosis

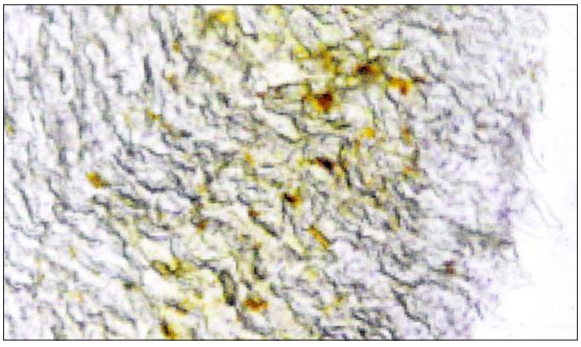

Fig. 2. C3 deposits in distal anastomosis

The C3 factor was found in the paraffin preparations obtained from both the proximal and distal anastomosis. The intensity of the presence of the C3 factor is statistically significantly different – the amount of C3 deposits is definitely bigger in the distal anastomosis than in the proximal anastomosis (Fig. 1, 2).

Electron microscope analysis

The research material was obtained during the necropsy performed after a six-month observation period. The specimens, examined using a JEM 100B electron microscope, were resected from the site of the proximal and distal anastomosis.

Proximal anastomosis

In the proximal anastomosis fibroblasts and fibrocytes were observed and only a few mastocytes (Fig. 3). There were multiple well-organised bundles of collagen fibres identified between the cells (arranged linearly along fibrocytes and mastocytes), making the main component of the extracellular matrix. Lack of macrophages, lymphocytes, plasma cells or granulocytes indicated that the inflammatory processes had no pathognomonic significance and that those inflammatory processes had subsided during the period of proximal anastomosis healing.

Distal anastomosis

Similarly as in the proximal anastomosis, the presence of fibroblasts and fibrocytes was observed. Additionally, several areas with numerous myofibroblasts were identified, some of which actively secreted collagen (Fig. 4). The

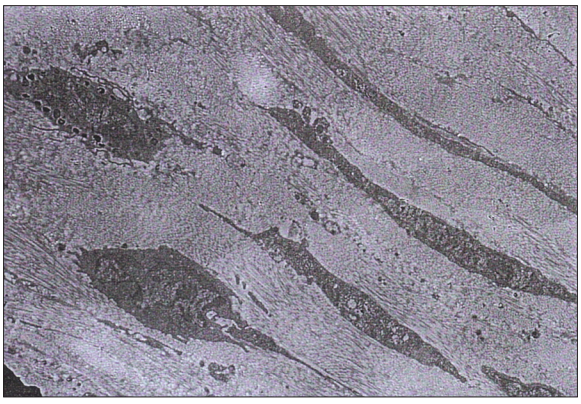

Fig. 3. Fibrocytes and mastocytes in the proximal anastomosis

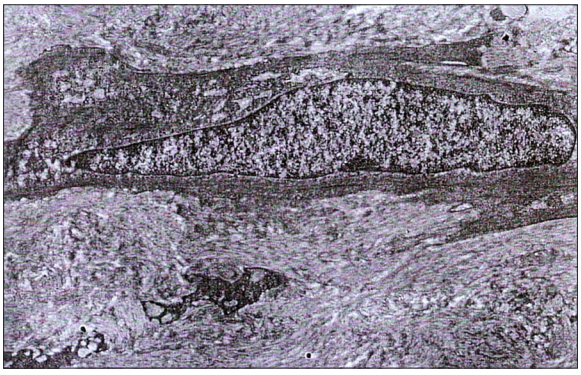

Fig. 4. Myofibroblast in the distal anastomosis

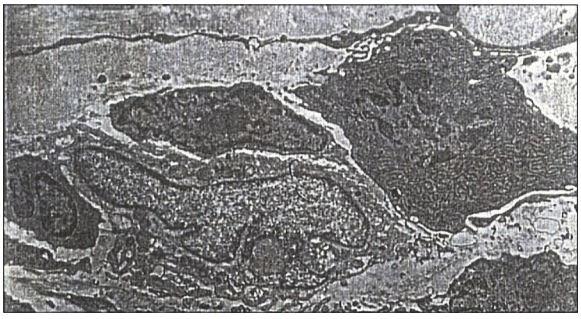

Fig. 5. Macrophages in the distal anastomosis

extracellular matrix contained a large number of collagen fibres formed in densely packed bundles. Also identified were several foci of numerous macrophages, plasma cells and segments of disintegrating cells. Myofibroblasts, which take part in healing of tissue loss and are found in granulation tissue, participate in the early and middle stage of wound healing and are lost when healing is completed, during the scar formation process. The occurrence of active macrophages and plasma cells together with the collagen, secreting myofibroblasts, indicates simultaneity of inflammatory processes and healing processes within the distal anastomosis. The picture observed in a distal anastomosis corresponds to a chronic inflammatory response, characterised by a high level of inflammatory cell activity (Fig. 5).

Discussion

Experimental works and clinical observations have shown that the ingrowth process of a synthetic vascular graft starts at the site of its anastomoses with the arterial system. The adsorption

of enzymatic proteins of the coagulation system and the complement on the anastomotic surface is the first phase of the body's humoral response to the presence of a prosthesis in the arterial system [1,10,14]. Over time, the activation of the complement proteins is gradually reduced. At the same time, there is an increase in activity of inflammatory cells within the anastomosis of the graft with the arterial system, caused by the active complement components including the C3 component [13,22,26]. This process is a manifestation of the involvement of macrophages, myofibroblasts and plasma cells in the reorganisation of mural thrombus into the neointima in the course of a cellular immune reaction [3,19,24].

In the case of prolonged activation of the enzymatic proteins of the complement and the inflammatory cells, there is constant progression of hypertrophic and regressive changes, which are associated with a high risk of clinical complications [7,11].

The results of immunohistological assays and electron microscope examinations have documented the increased activity of the complement system and the inflammatory cells: macrophages and myofibroblasts in the distal anastomosis. A negative effect of this phenomenon is persisting phagocytic and proteolytic activity of macrophages [5,6,17,25]. These processes may lead to re-exposure of the prosthesis fibres and their secondary contact with the coagulation system factors. This may promote thrombotic complications [12,17].

Re-exposed fibres may be infiltrated by bacterial cells; this process can lead to infection of the implanted vascular graft [7,8,11].

Another adverse change is the significant reduction of resistance to mechanical deformations of the vascular graft wall at its junction with the artery. A clinical expression of such a change is the formation of aneurysms at the level of distal anastomoses [7,8,11].

Proteolysis of the extracellular matrix is not the only negative consequence of the active complement and inflammatory macrophages in distal anastomoses. Macrophages and plasmocytes along with endothelial cells stimulate the myofibroblasts present in the connective tissue to an increased constant production of collagen [15,21]. A result of this process is a significant overgrowth of the inner "neointima" layer. In extreme cases this process promotes narrowing of the internal vessel diameter [16,20].

In the presented material, the results from transmission electron microscopy examinations of the anastomosis regions confirm the constant reorganisation of the extracellular matrix of the internal "neointima" layer. The results directly demonstrate the presence of macrophages, plasmocytes and increased production of collagen by myofibroblasts settled at the sites of distal anastomoses. It should be emphasised that the presence of macrophages and myofibroblasts has only been revealed in the distal anastomosis. This situation results from constant stimulation of the inflammatory cells by a significant increase in blood flow disturbances in the distal anastomosis compared to blood flow conditions in the proximal anastomosis [17,25,27].

The increase in forces acting on the walls of the artery and the vascular graft in the distal anastomosis is responsible for complement activation and the constant activation of the inflammatory cells. It is manifested in the histological image found in distal anastomoses, corresponding to a chronic inflammatory reaction [4,15,25,26,27].

The identification of differences in the presence of macrophages and myofibroblasts and the presence of the C3 component between the anastomoses is the original achievement of the present study. In the available literature, no such significant differences have been shown so far between the proximal and distal anastomosis in the humoral and cellular immune response caused by the presence of an artificial vessel in the arterial system. There are no published works concerning the possible clinical consequences of long-term activation of the inflammatory cells in the distal anastomosis. In the present study this was possible owing to the correlation of the obtained results with data published in the literature [7,11,15,20,21,23,25,27].

The present study has shown the differences concerning the presence of the C3 factor in the proximal and distal anastomosis in a vascular graft, as well as the differences in the location of the inflammatory cells within the anastomoses. The fibrocytes prevail in the proximal anastomosis. There are no active inflammatory cells in the proximal anastomosis. Active inflammatory cells, macrophages and myofibroblasts are present in the distal anastomosis.

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The authors have no potential conflicts of interest to declare.