ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXII, N 4, 19 SECTIO DDD 2009

[1] Chair and Department of Biochemistry, [2] Department of Vascular Surgery and Angiology,
[3] Department of Clinical Patomorphology, Medical University of Lublin

ANNA BELCARZ[1], STANISŁAW PRZYWARA[2], JACEK WROŃSKI[2],
GRAŻYNA GINALSKA[1], JUSTYNA SZUMIŁO[3],
ELŻBIETA KOROBOWICZ[3]

Nanobiomodification of vascular suture with vascular endothelial growth factor (VEGF) – in vivo preliminary study

Nanobiomodyfikacja nici szewnych za pomocą czynnika wzrostu śródbłonka naczyniowego (VEGF) – badania pilotażowe *in vivo*

Vascular endothelial growth factor (VEGF) is reported to play a very important role in angiogenesis and regulation of endothelial permeability [2, 6]. VEGF induces survival of endothelial cells, maintains the integrity of the vessel and contributes to the repair of the vascular wall [13].

Surgical dissection, exposure of the artery, transsection of the vessel and positioning of vascular clamps leads to a rupture of the adventitial vasa vasorum, which causes prolonged local hypoxia at the anastomotic site [1]. Moreover, the placement of vascular sutures obviously induces significant injury to the arterial wall.

To our knowledge, only a few scientific reports are available regarding the role of artificially delivered VEGF in the process of healing of vascular anastomosis [9, 12]. Infanger et al. [9] showed that intraluminal application of VEGF is beneficial to the healing process in vascular microsurgery in rats. Similarly, Seipelt et al. [12] proved that topical administration of VEGF in the line of vascular suture in rabbits maintains the luminal integrity by decreasing the level of fibrosis and calcification, thus enhancing the healing processes.

Based the on abovementioned findings we hypothesized that the biomodification of the surface of vascular suture with VEGF may enhance *in situ* healing of arterial wall and endothelium after placement of the suture. Our team has significant experience in modification of different biomaterials (used in vascular surgery and orthopedics) for antibiotic binding. These experiments allowed to obtain modified vascular prostheses [7] and hydroxyapatite granules [3], revealing durable antibacterial activity. Our research also concerns the modification of biomaterials to obtain increased cytocompatibility, neoangiogenesis and healing. In case of vascular sutures we intended to enhance the healing potency of these biomaterials, specially that this topic is problematic and still unexplored. In this article we describe the healing properties of VEGF-nanomodified sutures tested *in vivo* in rabbit model.

MATERIAL AND METHODS

Materials. Recombinant rat $VEGF_{164}$ and goat anti-rat VEGF antibody were supplied from R&D Systems, USA. Polyester silicon -coated PremiCron® 5/0 braided sutures were provided by B. Braun Melsungen AG, Germany.

PremiCron® sutures modification and VEGF attachment. 24 pieces (15 cm) of suture were modified by thermally polymerized 4-(1H-pyrrol-1-yl)benzoic acid (50 mg/ml aq. solution, 3 times, 15 min., 60°C), washed with distilled water and sterilized with ethylene dioxide. Among them, 14 pieces were subjected to 1h (4°C) incubation (under gentle shaking) with sterile 10 μg/ml VEGF solution in PBS (phosphate buffered saline) containing 0.2% bovine serum albumin (BDH, UK) and 5% glycerol (previously sterilized by microfiltration using Stericup, Millipore, USA), subsequently transferred to sterile vials, frozen and stored at –20°C (sutures A). 10 remaining modified pieces were left without further modifications being a control to the ones modified with VEGF (sutures B). The other 4 sutures were not modified in any way and served as an additional, "sham" control (sutures C).

In vitro detection of VEGF release from on PremiCron® sutures. 5-mm fragments of sutures were soaked for 1, 2 or 3 days in 0.25 ml sterile buffer A (20 mM phosphate buffer pH 7.4) in 96-wells flat bottom plates. 100 μl samples were analyzed for VEGF presence using ELISA test with goat antibody against rat VEGF with dilution 1:50 for 1 hour (R&D Systems, USA), Vectastain Elite ABC kit (Vector Laboratories, USA) and 3',3-diaminobenzidine tetrahydrochloride (DAB) as a chromogene according to manufacturer instructions. Calibration curves were obtained for the same VEGF that was attached to the examined sutures.

Animals, surgical procedure and follow up. 14 adult rabbits (body weight 2.5–3.5 kg) were selected for the study. Animals were maintained on the standard rabbit chow and water *ad libitum* and housed in a laboratory room with controlled circadian night/day cycle. The protocol was approved by the Local Ethic Committee for Animal Studies. In every rabbit both groins were opened and femoral arteries exposed. Bilaterally femoral arteries were incised axially and a single hand suture vascular repair was performed. The arteries were sutured as described in Table 1.

Table 1. Experimental design

Group	Treatment	Number of animals	Artery	Time when animal was sacrificed (days)
A	rrVEGF/modified suture	14	left	14
B	modified suture (control I)	10	right	14
C	non-modified suture (control II)	4	right	14

Rabbits were anesthetized by intraperitoneal application of chloral hydrate (30–40 mg/kg i.p.). The skin in the area of groins was shaved and swabbed with povidone-iodine solution and then incised over the femoral artery. Femoral artery was carefully dissected, microclamps were positioned proximally and distally enabling placement of the suture within the 2 cm segment of artery between the clamps. Before clamping, a single dose of Enoxaparinum sodium was given subcutaneously (1mg/kg s.c.). Single PremiCron® suture (sutures A, B, C) was used for vascular repair of axially incised arterial wall and then the clamps were released. Blood loss was minimal in every case. Subsequently, the patency of the artery was confirmed by visual assessment, refilling and pulsation of the artery distally to the area of suture. Before the skin closure with 5/0 Nylon sutures, the surgical wound was washed up with topical administration of gentamicin.

The animals remained on 150 mg daily dose of acetylic acid during 14 days. No acute limb ischaemia or obvious clinical symptoms of chronic limb ischaemia were observed during the follow up. All animals were sacrificed on day 14 after surgery. To harvest *intra vitam* sutured femoral arteries, rabbits were intraperitonealy anaesthetised with sublethal dose of chloral hydrate (60–80 mg/kg i.p.). After the specimens were taken, animals were placed in CO_2 gas chamber and sacrificed.

Transmission Electron Microscopy (TEM). Transmission electron microscopy was performed according to the routine protocol. Harvested tissue specimens were immediately stored in glutaraldehyde solution. After fixation and postfixation in 2% OsO_4 solution (0.1 M cacodil buffer), the specimens were dehydrated in ascending alcohol series, embedded in Epon and cut on Reichert Ultracut (Reichert-Jung, Nussloch, Germany). For contrast, the 2% uranyl acetate/lead citrate together with Reynolds fluid were used. Specimens were analysed using EM 900 Zeiss microscope.

Histological and immunohistochemical examination. The specimens of harvested segment of femoral artery were fixed in 10% buffered formalin for 24 h. After routine processing to the paraffin blocks, 4 μm transverse slides were stained with hematoxylin and eosin (H+E) as well as resorcin-fuchsin and van Gieson's methods. The slides were investigated using light microscope (Olympus BX45, Japan).

Immunohistochemical technique was applied on paraffin sections with goat antibody against rat VEGF with dilution 1:50 for 1 hour (R&D Systems, USA), Vectastain Elite ABC kit (Vector Laboratories, USA) and 3',3-diaminobenzidine tetrahydrochloride (DAB) as a chromogene according to manufacturer directions. The appropriate positive and negative controls were also performed. The slides were investigated using light microscope (Olympus BX45, Japan).

RESULTS

VEGF RELEASE

Presence of VEGF on PremiCron® sutures failed to be confirmed by immunostaining for the technical reason. Analysis of samples obtained during the test of VEGF release from sutures showed that the amount of released factor was approx. 4 ng/cm suture/day; however, the values were close to the method detection limit. Therefore, the VEGF-modified sutures were implanted to rabbits to evaluate *in vivo* the possible effect of nano-amounts of the growth factor on wound healing process.

SURVIVAL AND PATENCY RATE

All animals survived the 14 days of observation. Neither acute limb ischaemia nor obvious clinical symptoms of chronic limb ischaemia were observed during the follow up. All harvested arteries remained patent.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Our preliminary evaluations of specimens containing VEGF (group A) by transmission electron microscopy showed the differences when compared with those from other groups. In group A we observed increased presence of collagen, a higher number of fibroblasts with widened rough endoplasmatic reticulum in comparison with both control groups (B and C). In granulation tissue, activated fagocitary cells were present and dividing cells were observed in intima media. Muscular layer was thickened. A lesser extent of vacuolisation and dystrophy of endothelium and smooth

muscles was noted. The suture seemed to be better incorporated in case of group A, surrounded by collagen and elastin fibers (Fig. 1). Lymphocytes, granulocytes and macrophages were more fagocitarilly active and additionally the presence of eosinophiles surrounding VEGF-modified sutures was detected.

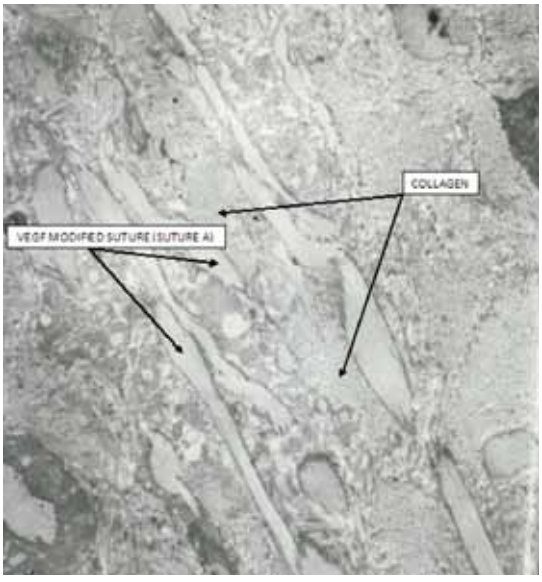

Fig. 1. Increased presence of collagen in transmission electron microscopy specimen from group A - VEGF coated sutures A (magnification x 3000)

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL OBSERVATIONS

Differences in histological appearance between all study groups were less unambiguous than TEM results. In most cases the arteries were slightly affected and mild focal thickness of intima with derangement of media of femoral artery were revealed. Both elastic laminas were preserved. Diffuse granulation tissue formation rich in endothelial cells, fibroblasts and capillaries (Fig. 2 A, B, C) were seen in all the animals. Sutures and areas of dystrophic calcification were usually surrounded by multinucleated giant cells – macrophages (Fig. 3 A). In some cases the fibrous tissue around the artery was composed of stellate and spindle-shaped cells embedded in myxomatous extracellular matrix. Furthermore, features of atrophy and vacuolar degeneration of skeletal muscles were also seen.

Also, the location and intensity of immunostaining for VEGF in tissue was similar in all rabbits regardless of the study group. A strong positive reaction was noted in endothelium of blood vessels as well as in dispersed cells of granulation tissue corresponding to endothelial cells (Fig. 2 D). Multinucleated giant cells, especially around threads were positive as well (Fig. 3 B). Immunostaining was also seen in the smooth muscle of blood vessels.

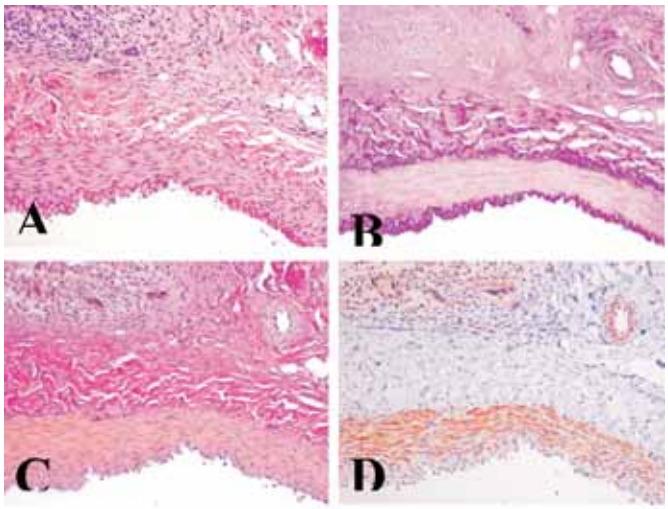

Fig. 2**.** Femoral artery surrounded by granulation tissue close to the area of suture A (A – hematoxylin and eosin; B – resorcin-fuchsin; C – van Gieson's stain; D – immnunohistochemical reaction against VEGF – ABC/HRP; lens magnification x10)

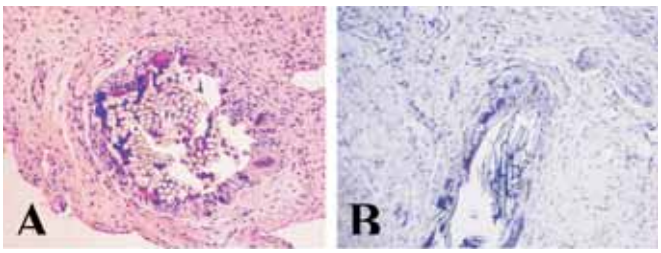

Fig. 3. Cross section of suture A (A – hematoxilin and eosin stain; B – immnunohistochemical reaction against VEGF – ABC/HRP; lens magnification x20)

DISCUSSION

Vascular endothelial growth factor (VEGF) is a potent mediator in angiogenesis and vasculogenesis, it functions in wound healing and its expression is significantly increased after vascular reconstructions in the vein grafts and vascular anastomosis, thus facilitating reendothelialisation [4, 8]. There are reports that topical or intraarterial, artificial delivery of VEGF in the area of vascular anastomosis can contribute to improved healing of vascular anastomosis thus preventing its thrombosis and formation of neointima, which may cause significant stenosis [9, 12]. However, the problem of such VEGF delivery system concerns its single application to the injury site because the factor delivered in this manner is usually quickly eluted from the application site. The attempts of immobilization of VEGF on biomaterials should be therefore made to obtain the modified biomaterials releasing its protein in a more sustained manner. Among described procedures, Petersen et al. [11] has described the method of Ethibond (Ethicon, Norderstedt, Germany) sutures coating with poly (d, l-lactide) (PDLLA) containing VEGF (method of entrapment). However, in their experiments the local application of VEGF via PDLLA-coated sutures did not promote meniscus healing. These sutures contained a huge

amount of VEGF (250 μg/suture). It was reported that such an excess of growth factor may be a reason for severe vascular leakage, hypotension due to its tendency to increase vascular permeability and overproduction of malformed and leaky vessels [5].

Results of the preliminary *in vivo* experiments gave partially encouraging results. Transmission electron microscopy suggested that the amount of collagen fibers was increased in group A in comparison with other experimental groups (Fig. 1). This finding is coherent with results of Infanger et al. [9], who demonstrated for the first time that VEGF significantly increases the amounts of several collagen types, which is positive in terms of microvascular remodeling. Moreover, in group A the processes of different cells activation were more pronounced when compared with other groups.

However, histological examinations did not show similarly clear differences between the analyzed animal groups. Sutures A (with VEGF) and B and C (without VEGF) evoked a similar reaction of artery wall and surrounding tissues. This finding is in agreement with results of Lindner [10] but in contrast to the majority of publications showing the beneficial effects of VEGF on healing in vascular injury. Immunostaining in all groups showed increased native VEGF production in endothelial cells, surrounding artery granulation tissue and smooth muscles. That has been already shown [6, 9] to be a typical vessel response to any type of vascular injury, in our case produced by the placement of vascular suture.

Although not all results obtained in our experiments suggest the beneficial effect of artificially delivered VEGF on healing after vascular injury, especially in histological analysis, the results of TEM seem to be more promising. They seem to confirm that nanoquantities of VEGF attached to modified sutures play a supporting role in wound healing process. Surely, the risk of harmful effect of exceeding amounts of VEGF additionally introduced to artery injury (as already mentioned in Discussion) is thus avoided in case of our nanomodified sutures. However, to increase the healing effect, we suppose that the amount of attached VEGF should be slightly larger to evoke the appropriate effects, visible in histological observations. Small concentrations of VEGF solution (10 μg/ml) used for the attachment procedure could be the reason for this discrepancy. Therefore, further optimization of the amount of VEGF bound to sutures is necessary. Moreover, the overexpression of native VEGF in artery injury could have suppressed the effects of VEGF artificially delivered on the suture. This possibility should also be considered.

On the basis of the obtained results it is obvious that further more detailed multi-aspect investigations are required to finally establish the optimized VEGF-coated suture for best healing of arterial anastomosis. On the other hand, our nanotechnological coatings of sutures surface did not produce any histologically apparent tissue reactions different to those caused by unmodified sutures. This preliminary finding shows that our biosurface modification does not evoke the toxic side effects and may be safely used in further experiments on animals.

CONCLUSIONS

We performed the first, relatively stable, nanobiomodification of vascular suture with VEGF. Preliminary, transmission electron microscopy results (on a cellular level) are promising in terms of improved healing of vascular anastomosis using VEGF bionanomodified sutures. The suture surface modification tested in the experiments did not reveal any, at least histologically visible, toxicity when compared to unmodified sutures. Therefore, we hope that our new biocompatible, nanotechnologically modified surface could be used as a platform for future binding of pharmacologically and biologically active molecules to surgical sutures.

Authors thank immunochemistry and transmission electron microscopy specialists: Mrs Mariola Bigas, Mrs Maria Nowak, Mrs Teresa Agnieszka Karpińska MSc. and Dr Mirosław Łańcut for their technical and organizational support. We also thank Dr Marek Iłżecki and Dr Jan Jakub Kęsik for their surgical support during the prostheses implantation.

REFERENCES

1. Barker S. G., Talbert A., Cottam S. et al.: Arterial intimal hyperplasia after occlusion of the adventitial vasa vasorum in the pig. Arterioscler. Thromb., 13(1), 70, 1993.
2. Bates D. O., Harper S. J.: Regulation of vascular permeability by vascular endothelial growth factors. Vascul. Pharmacol., 39 (4-5), 225, 2002.
3. Belcarz A., Ginalska G., Zalewska J. et al.: Covalent coating of hydroxyapatite by keratin stabilizes gentamicin release. J. Biomed. Mater. Res. Part B. Applied Biomaterials, 89B, 102, 2009.
4. Byrne A. M., Bouchier-Hayes D. J., Harmey J. H.: Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). J. Cell. Mol. Med., 9 (4), 777, 2005.
5. Drake C. J., Little C. D.: Exogenous vascular endothelial growth factor induces malformed and hyperfused vassels during embryonic neovascularization. Proc. Natl. Acad. Sci. USA, 92, 7657, 1995.
6. Ferrara N.: The role of VEGF in the regulation of physiological and pathological angiogenesis. EXS, 94, 209, 2005.
7. Ginalska G., Osińska M., Uryniak A. et al.: Antibacterial activity of gentamicin-bonded gelatin-sealed polyethylene terephthalate vascular prostheses. Eur. J. Vasc. Endovasc. Surg., 29, 419, 2005.
8. Hamdan A. D., Aiello L. P., Misare B. D. et al.: Vascular endothelial growth factor expression in canine peripheral vein bypass grafts. J. Vasc. Surg., 26 (1), 79, 1997.
9. Infanger M., Shakibaei M., Kossmehl P. et al.: Intraluminal application of vascular endothelial growth factor enhances healing of microvascular anastomosis in a rat model. J. Vasc. Res., 42 (3), 202, 2005.
10. Lindner V., Reidy M. A.: Expression of VEGF receptors in arteries after endothelial injury and lack of increased endothelial regrowth in response to VEGF. Arterioscler. Thromb. Vasc. Biol., 16 (11), 1399, 1996.
11. Petersen W., Pufe T., Stärke Ch. et al.: The effect of locally applied vascular endothelial growth factor an meniscus healing: gross and histological findings., Arch. Orthop. Trauma. Surg., 127, 235, 2007.
12. Seipelt R. G., Backer C. L., Mavroudis C. et al.: Topical VEGF enhances healing of thoracic aortic anastomosis for coarctation in a rabbit model. Circulation, 108 Suppl. 1, II 150, 2003.
13. Zachary I.: Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor Am. J. Physiol. Cell. Physiol., 280 (6), C1375, 2001.

SUMMARY

A method of quite stable binding of Vascular Endothelial Growth Factor (VEGF) to vascular suture has been developed. The effect of nanobiomodified suture on the healing process of sutured artery wall was evaluated *in vivo* in rabbit model, using histopathological, immunohistochemical and transmission electron microscopy methods. Results of transmission electron microscopy showed beneficial effects of artificial VEGF-coated sutures on the healing process. The presented VEGF-coated sutures improved the healing process and biomodified coatings did not produce histological appearance of biological toxicity or unusual tissue reactions.

STRESZCZENIE

Opracowano metodę relatywnie stabilnego wiązania Czynnika Wzrostu Śródbłonka Naczyniowego (VEGF) do nici szewnych. Wpływ nanobiomodyfikowanej nici na proces leczenia przeszywanej ściany arterii został oceniony *in vivo* w modelu króliczym z użyciem technik histopatologicznych, immunohistochemicznych i elektronowego mikroskopu transmisyjnego. Rezultaty uzyskane z zastosowaniem mikroskopii transmisyjnej wykazały korzystny wpływ nici modyfikowanych VEGF na proces gojenia. Nici modyfikowane VEGF wzmogły proces gojenia, a zastosowane biomodyfikacje nie wywołały toksyczności biologicznej ani nietypowych zachowań tkanek w obrazie histologicznym.