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IMMUNOHISTOCHEMICAL STUDY OF THE EXTRACELLULAR MATRIX FORMED DURING PERIPHERAL NERVE REGENERATION THROUGH A KNITTED PROSTHESIS

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A b s t r a c t

The extracellular matrix (ECM) formed in a knitted prosthesis, which was applied between the stumps of a transected peripheral nerve, was investigated to find out whether it provided suitable conditions for nerve regeneration. A knitted, polyethylene terephthalate (PET) silk tube, filled with Gelaspon®, was used to guide outgrowth of the transected sciatic nerve in the rat. After 6 weeks, the prosthesis contained a nerve outgrowth connecting the nerve stumps. Cryostat cross-sections through the middle of the nerve prosthesis, including its content, were analysed immunohistochemically for the presence of selected ECM molecules (thrombospondin, fibronectin, chondroitin sulfate A and C, type IV collagen, and vitronectin) that are known to play a role in axonal growth and maturation. The immunohistochemical staining revealed that thrombospondin, fibronectin, chondroitin sulfate A and C, and type IV collagen were localised in the connective tissue surrounding the nerve outgrowth, in the perineurium of individual minifascicles, as well as in the endoneurium among regenerated nerve fibres. The immunoreaction for thrombospondin was more intense in the perineurium than in the endoneurium. No immunostaining for vitronectin was observed in cross-sections through the nerve outgrowth. Our results suggest that the environment formed during nerve regrowth within a knitted prosthesis provides conditions supporting the regeneration of nerve fibres.

K e y w o r d s

Regeneration, Peripheral nerve, Extracellular matrix, Immunohistochemistry

INTRODUCTION

Peripheral nerve injury is a frequent consequence of trauma or surgical removal of tumours. Larger peripheral nerve defects are usually repaired by microsurgical implantation of grafts harvested from cutaneous nerves. However, clinical results of functional re-innervation are still unsatisfactory. In addition, the patient is exposed to further trauma when the nerve graft is acquired. For these reasons, alternative methods of bridging a gap between the stumps of an injured nerve have been sought. One of them is based on „entubation“, i.e., a procedure by which both stumps of a severed nerve are inserted into a tube guiding the regenerating axons through the gap.

Silicone chambers (tubes) are used as a standard experimental model to study cellular and molecular changes during the nerve regeneration process (*12, 26, 35, 42).* Within the first few hours of implantation, the chamber is filled by a fluid enriched with neurotrophic and neurotropic molecules *(25, 29*) and factors that stimulate migration and proliferation of Schwann cells (*23).* During the 1st week, an acellular fibrous matrix replaces the chamber fluid content. The fibrin matrix formation is crucial for the migration of Schwann cells and, consequently, for regrowth of axons through the chamber (*37*). Fibroblasts, Schwann and endothelial cells migrate into the matrix from both nerve stumps within 2 weeks from implantation (*27, 33, 35, 40*). By 3 weeks, axons grow from the proximal stump and, at the beginning of the 4th week after nerve entubation, reach the distal stump over a 10-mm long distance. Subsequently, the regenerated axons enlarge in their diameter and their myelination proceeds in a proximal-to-distal direction (*15, 22, 35).*

It has been found that, when the chamber is filled with phosphate buffered saline (PBS) at the time of implantation, the formation of a nerve outgrowth increases in comparison with implantation of an empty chamber (*36).* Exogenous fibrin precursors or artificial fibrin sponge packed into the silicone chamber provide a suitable matrix for an enhanced migration of Schwann cells and subsequent regrowth of axons (*14, 15, 37, 38*). In our experimental chamber model, we have used a resorbable artificial fibrin sponge (Gelasponģ) soaked with PBS or insulin to improve substrate conditions and to test the migration capacity of Schwann cells (*12, 13).*

However, nerve conduits with smooth, rigid walls prepared, for instance, from silicone tubes have some imperfections. One of them is compression of the regenerated nerve segment during flexion of the prosthesis, which may result in axon damage. A solution of the problem may be based on the use of prosthesis with soft and corrugated walls for reconnection of the nerve stumps. Our previous experiments have revealed that a knitted silk tube with a corrugated wall, routinely used as an artificial vascular graft, has the properties required to avoid any compression of regenerating nerve fibres (*19*).

ECM molecules are involved in many aspects of cell signalling during neuronal differentiation, neuroblast migration, the stimulation and guidance of nerve fibre outgrowth, and Schwann cell proliferation and the myelination of peripheral nerves (*2, 3, 5).* Some of the ECM components have been shown to influence the growth and maturation of regenerating axons after injury (*8, 18, 20*).

The aim of the present study was to investigate whether ECM conditions provided in knitted prostheses would be suitable for nerve regeneration. The presence of several ECM molecules in the nerve outgrowth, namely, thrombospondin, fibronectin, chondroitin sulfate, collagen IV, and vitronectin, known to play a role in cell migration and axonal growth and maturation, was analysed by an immunohistochemical method.

MATERIALS AND METHODS

The experiments were performed on 12 female Wistar rats weighing about 200-250 g (AnLabLtd.). The animals were handled according to the ethical laws governing the protection of experimental animals at the Faculty of Medicine, Masaryk University, Brno, Czech Republic. Knitted tubes made of PET silk were produced by the Hosiery Research Institute in Brno. The tubes were 2 mm in inner diameter and 14 mm in length; their walls were corrugated but for the end parts that were straight (about 2 mm), which facilitated the insertion and fixation of nerve stumps. The prostheses were sterilised by gamma irradiation before delivery.

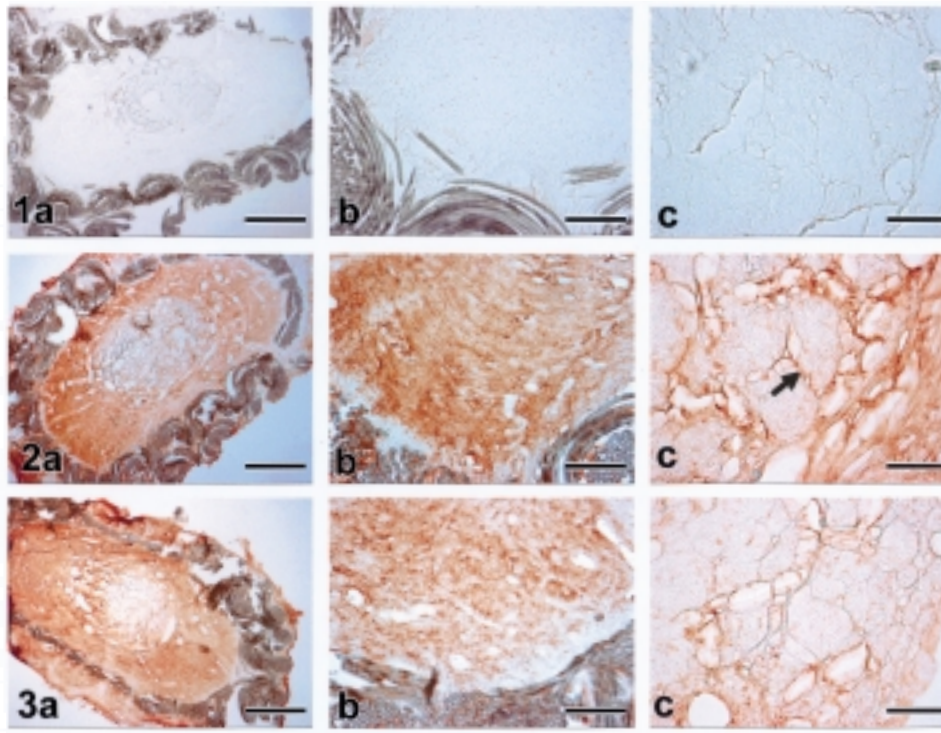
Rats were anaesthetised by intraperitoneal injection of a mixture (0.2 ml/100g) containing xylazine (4mg/ml) and ketamine (40 mg/ml). Before implantation, prostheses were filled with Gelaspon® (Ankewerke, F.R.G.) soaked with phosphate buffered saline (pH 7.2). After skin incision, the sciatic nerve was exposed and cut in the middle position of the thigh. A knitted prosthesis filled with Gelaspon® was inserted between the stumps of the transected sciatic nerve and fixed with two epineurial sutures (Ethicon 10/0), leaving a 10 mm gap.

The animals were sacrificed by inhalation of an overdose of ethyl ether after 6 weeks and perfused transcardially with warm PBS (37°C) and then Zamboni's fixative (39). The prostheses containing regenerated nerve segments were removed and immersed in Zamboni's fixative solution overnight. The samples were washed in PBS containing 10% sucrose overnight and cryostat cross-sections, 10-mm thick, were cut through the middle of the prosthesis and its content. The indirect immunohistochemical method was used for detection of thrombospondin, fibronectin, chondroitin sulfate A and C, collagen IV, and vitronectin molecules in the cross-sections of nerve outgrowth.

Briefly, the sections were treated with TRIS-NaCl (pH 7.4) containing 0.3 % bovine serum albumin and 0.3 % Triton X-100 for 15 minutes. Then, endogenous peroxidase activity was inhibited with TRIS-NaCl (pH 7.4) containing 10% methyl alcohol and 10% H_2O_2. After washing in TRIS-NaCl, sections were incubated with primary antibody in a humid chamber for 3 hrs. Information regarding the primary antibodies used in this study, including working dilutions and sources, is summarised in *Table 1*. The binding of the primary antibody with an antigen was visualised by an EnVision Kit (Dako, Denmark) according to the instructions. After three washes in TRIS-NaCl (pH 7.4), the sections were developed in DAB solution (50 mM TRIS-HCl buffer pH 7.4 containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride) supplemented with 0.001% H_2O_2. Finally, the sections were washed, dehydrated in a graded alcohol series and mounted in Eukitt®. Control sections were immunostained with an omission of the primary antibody. The immunohistochemical staining was observed and documented in a Leica DMBL light microscope equipped with a DC 100 camera.

Table 1
Antibodies used for immunohistochemical analysis

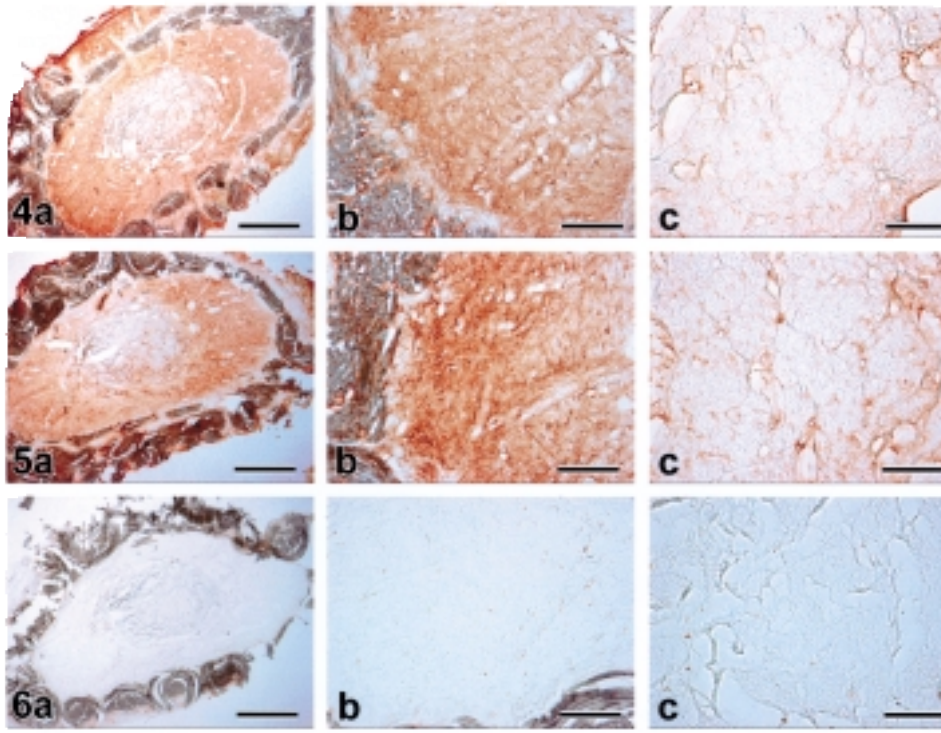
LIGAND	ANTIBODY	SOURCE	DILUTION
Thrombospondin	mAb mouse	Immunotech	1:50
Fibronectin	mAb mouse	Sigma	1:200
Chondroitin sulfate	mAb mouse	Sigma	1:200
Collagen IV	mAb mouse	Sigma	1:500
Vitronectin	pAb goat	Santa Cruz	1:500



Bar, 83 μm Bar, 20 μm Bar, 10 μm

Figs. 1–6

Results of immunohistochemical staining observed in cross-sections through knitted tubes including nerve outgrowth. Different structures were revealed in relation to the magnification used: 83 μm (a), wall of the tube with the entire nerve outgrowth; 20 μm (b), connective tissue between the centrally located minifascicles of axons and the tube wall; 10 μm (c), details of minifascicles in the centre of the nerve outgrowth. Control sections were incubated without primary antibody (1a,b,c). Immunohistochemical reaction showed thrombospondin in connective tissue at the surface of nerve outgrowth (2a,b) as well as inside axon minifascicles; perineurium (arrow) is more distinct than endoneurium (2c). The results of immunostaining for fibronectin were similar to those for thrombospondin (3a,b) only minifascicles were more diffuse (3c). Positive reactions to chondroitin sulfate A and C and type IV collagen were seen in connective tissue surrounding the nerve outgrowth inside the prosthesis (4a, 5b) as well as in the perineurium separating newly formed minifascicles (4b,5b). Inside minifascicles, the presence of chondroitin sulfate A and C and type IV collagen was detected in the endoneurial space among nerve fibres (4c, 5c). No immunoreaction to vitronectin in the nerve outgrowth was seen at any level of detection (6a,b,c).



Bar, 83 μm Bar, 20 μm Bar, 10 μm

Figs. 1–6 (4a to 6c)

RESULTS

The regenerative capacity of the sciatic nerve supported by a modified vascular knitted prosthesis in the rat was comparable to that observed in our previous experiments on tibial and median nerves in the rabbit (*19*).

At 6 weeks after implantation, each prosthesis contained an outgrowth of the regenerated nerve connecting the proximal and distal nerve stumps. The corrugated walls of the prosthesis were covered by mesothelial cells to protect the regenerating axons from growing out of the tube. Transverse cryostat sections through the prosthesis and its content revealed the regenerated nerve outgrowth in the central position of the tube lumen. The nerve outgrowth consisted of regenerated nerve fibres organised into distinct minifascicles separated from each other by the perineurium.

The space between the nerve outgrowth and the prosthesis wall was filled with a thick layer of connective tissue containing blood vessels and capillaries. The blood vessels were also observed in the mesothelium at the outer surface of the prosthesis.

Control sections, which were incubated without the primary antibody, displayed no brown staining *(Figs. 1a,b,c).* Immunohistochemical staining with the corresponding primary antibodies revealed the presence of thrombospondin, fibronectin, chondroitin sulfate A and C, and type IV collagen in the connective tissue surrounding the nerve outgrowth inside the prosthesis *(Figs. 2a,b,3a,b, 4a,b,5a,b).* A positive immunoreaction to these ECM molecules was also observed in the perineurium separating newly formed minifascicles in the nerve outgrowth. Inside the minifascicles, the presence of these molecules was detected in the endoneurial space among nerve fibres (*Figs. 2c,3c,4c,5c*). Intensity of the immunoreaction to thrombospondin was higher in the perineurium than in the endoneurium of the minifascicles *(Figs. 2c,* arrow*).* Immunostaining for vitronectin was not observed in any part of the nerve segment regenerated within the knitted prosthesis *(Figs. 6a,b,c).*

DISCUSSION

Entubation is one of the alternative methods to span a peripheral nerve defect. Both stumps of the severed nerve are fixed into the ends of a tubular prosthesis that serves as a bridge over the nerve gap. Neurotrophic and neurotropic substances produced by cells of the distal stump are accumulated inside the tube and create a milieu stimulating growth of axons from the proximal nerve stump. This offers an advantage over the use of graft because there are no other influences restricting regenerating axons in their growth. The regrowing axons are probably „navigated" to the appropriate peripheral stump fascicles by neurotropic factors. It was experimentally demonstrated that the smaller the gap between stumps of the severed nerve, the lesser the risk of incorrect orientation of axons (*17*).

Tubes prepared from various materials, both natural and synthetic, have been used for entubation (*6, 9, 11, 31, 32*). Silicone tubes are the most usual experimental models to study molecular and cellular processes during peripheral nerve regeneration. They isolate the gap space between nerve stumps from the adjacent tissues and prevent the exudate important for axonal growth stimulation from its leakage into the surroundings. The fact that the composition of an exudate inside the silicone tube can be both analysed and changed is another advantage of the prosthesis. On the other hand, there is a disadvantage in that its smooth, rigid wall has a potential for deformation and, therefore, the subsequent compression of a regenerated nerve segment may occur.

We have reported previously that the problem of nerve compression inside the prosthesis can be solved by the use of a knitted tube with a corrugated wall,

routinely used as a vascular graft (*19).* In contrast to the findings on nerves regenerated through a silicone tube, as reported by Le Beau (*22),* we did not find any manifestation of nerve outgrowth compression caused by the flexibility of the PET knitted prostheses with corrugated walls used in the rat experiments.

In adult vertebrates, regeneration is much more successful in the peripheral nervous system than in the CNS. The results of recent studies have suggested that this discrepancy reflects not the capacity of particular groups of neurons to regenerate, but the different environments of peripheral and central neurons. Differences in ECM are likely to be a major reason for the disparity in regeneration between peripheral and central nervous systems (*7*).

Our results based on immunohistochemical detection revealed that the molecules known to provide stimulating conditions for axon growth were present, as EMC components, in the outgrowth of nerves regenerating through knitted prostheses.

Thrombospondin was present in the ECM surrounding the regenerated nerve segment as well as in the perineurium of new fascicles of axons. These findings are in agreement with the conclusion that thrombospondin stimulates growth of axons during regeneration of the peripheral nerve (*18*).

Fibronectin was also found on the surface of the nerve outgrowth as well as in the perineurium of the nerve fascicles in the knitted prosthesis. Fibronectin is an ECM molecule contributing to the formation of fibrin fibres by exudate polymerisation during the first days after nerve guide implantation (*38, 41*). The fibrin fibres, oriented in the longitudinal direction, stimulate the migration of Schwann cells that, subsequently, promote the growth of regenerating axons (*1*).

The findings concerning the effects of chondroitin sulfate proteoglycans on growing axons are rather controversial (*21, 24).* Generally, glycosaminoglycans alone or during interaction with some other molecules (e.g., fibronectin) are described to modulate the environment for post-traumatic tissue remodelling and axonal regrowth (*4, 8*). This could be an explanation for the presence of chondroitin sulfate A and C in the nerve regenerated through a knitted prosthesis.

Type IV collagen is a regular component of the basal laminae. Correspondingly, immunostaining for collagen IV was found in the perineurium of minifascicles as well as in the endoneurium of the nerve outgrowth in the prosthesis related to the basal laminae of regenerated fibres. Surprisingly, collagen IV immunostaining was also demonstrated in the connective tissue matrix distributed between the nerve outgrowth and the prosthesis wall. The localisation of collagen IV in connective tissue surrounding the nerve outgrowth could be related to its soluble form (*10*) or to its intracellular localisation in the cells not covered by the basal lamina (*30*).

Vitronectin is an important adhesive glycoprotein of the blood plasma that also regulates cell-mediated proteolytic enzyme cascades, including the complement,

coagulation, and fibrinolytic systems (*34*). In the nervous system, vitronectin promotes migration of astrocytes (*16*) while it does not stimulate migration of Schwann cells *in vitro* (*28*). The latter view was supported by the fact that no immunohistochemical staining for vitronectin was found throughout the nerve outgrowth in the knitted prosthesis. Our results of immunohistochemical staining suggest that the ECM, which is present in regenerating nerves outgrowing through knitted prostheses, is composed of molecules known to provide stimulating conditions for nerve regeneration.

It can be concluded that knitted prostheses are more suitable for guiding regenerating axons than tubes with rigid walls and that the composition of ECM which develops in knitted prostheses is favourable for nerve regeneration.

A c k n o w l e d g e m e n t

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IMUNOHISTOCHEMICKÁ ANALÝZA EXTRACELULÁRNÍ MATRIX VYTVOŘENÉ BĚHEM REGENERACE PERIFERNÍHO NERVU V PLETENÉ PROTÉZE

S o u h r n

Pletená protéza vyrobená z PET hedvábí s prohýbaným povrchem byla použita ke spojení pahýlů proťatého sedacího nervu laboratorního potkana. Pletená tuba byla k pahýlům nervu fixována suturou tak, aby byly konce pahýlů vsunuty do tuby a plochou řezu byly v kontaktu s Gelasponem napuštěným fyziologickým roztokem, který byl před aplikací vložen do tuby. Po 6 týdnech přežívání obsahovala protéza regenerovaný nervový segment, který spojil oba nervové pahýly. Příčné kryostatové řezy vedené středem tuby s jejím obsahem odhalily přítomnost regenerovaných axonů uspořádaných do malých svazečků, které byly odděleny perineuriem. Nebyl pozorován únik axonů z nitra protézy přes její stěnu. Imunohistochemická analýza prokázala přítomnost některých molekul extracelulární matrix, o kterých je známo, že mají význam pro růst a diferenciaci axonů (trombospondin, fibronektin, chondroitinsulfát A a C, kolagen typu IV). Pozitivní imunoreakce na uvedené molekuly byla pozorována ve vazivu, které vyplňovalo prostor mezi nervovým regenerátem a vnitřním povrchem protézy, v perineuriu i v endoneuriu mezi jednotlivými axony. Imunoreakce na trombospondin byla výraznější v perineuriu než v endoneuriu. Přítomnost vitronektinu nebyla imunohistochemicky prokázána v žádném regenerovaném úseku nervu. Získané výsledky ukazují, že po aplikaci pletené protézy mezi pahýly přerušeného periferního nervu se uvnitř protézy vytvářejí vhodné podmínky pro regeneraci nervu. Zvlněné stěny tohoto typu nervové protézy nezpůsobují kompresi nervu, která je často pozorována při regeneraci nervu v tubě s rovnou stěnou.

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