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From Cell Biology to Tissue Engineering

Assessment of immunologic, proangiogenic and neurogenic properties of human peripheral nerve epineurium for potential clinical application

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Summary. The epineural sheath is a promising naturally occurring material for enhancement of peripheral nerve regeneration. Based on a literature search there is a limited number of reports on the biological and immunological properties of human epineurium. The goal of this study was to assess, using immunocytochemical methods, the immunological (HLA class I and II antigens, T lymphocytes, macrophages), proangiogenic (VEGF, CD31), and neurogenic (GFAP, S-100) properties of human epineurium isolated from ilioinguinal nerves (n=19) taken from deceased donors, and from sciatic nerves (n=12) taken from limbs amputated due to critical ischemia. Our studies confirmed reduced expression of HLA class II antigens on the infiltrating cells, a reduced number of T lymphocytes, and greater vessel density in the epineurium obtained from deceased organ donors. Macrophages were more abundant in the epineurium isolated from the amputated limbs. We found that the epineurium harvested from peripheral nerves of the deceased donors showed negligible immunogenic and increased proangiogenic properties compared to the epineurium of nerves taken from amputated limbs. These findings support the rationale to use human epineurium obtained from deceased donors as a new biological material for enhancement of peripheral nerve repair for potential clinical application in regenerative medicine.

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Introduction

The term ‘epineurium’ appeared in the nineteenth century (Key and Retzius, 1873), along with terms for other connective tissues surrounding peripheral nerve fascicles - perineurium and endoneurium. The construction and physiological functions of the epineurium have been widely examined and thoroughly described, but the immunological properties of the epineurium are still poorly characterized.

The epineurium as the outermost sheath is a continuation of the dura mater in peripheral nerves (Topp and Boyd, 2006). Its major components are collagen type I and type III fibrils, while elastin fibers are a minor component. These fibrillar structures are arranged in flat layers running along the nerve trunk and demonstrate a regular dense, wavy pattern which is crucial in maintaining biomechanical properties of the whole nerve bundle during tension (Thomas, 1963; Stolinski, 1995). Additionally, there is often present an adipose component, which plays the important role of protection from compression and tension (Millesi et al., 1995); it contains fatty acids indispensable for supporting the metabolism of axons and Schwann cells and as an endocrine tissue also maintains homeostasis in the endoneurial area (Saltiel and Kahn, 2001). On the inner side the epineurium contacts directly with the perineurium, and this association is close, without the

possibility of mechanical separation (Stolinski, 1995).

The epineurium also creates a matrix for blood vessels which supply all cells involved in proper function of nerves (Saltiel and Kahn, 2001). Interestingly, lymphatic drainage occurs only within the epineurium, without the entire endoneurial areas (Dyck et al., 1993). There are also scattered fibroblasts, mast cells and macrophages transmigrated from the vessels (Grant et al., 1999).

The peripheral nervous system has remarkable abilities of regeneration after injury. However, successful regeneration depends on both injured axons and non-neuronal cells, including Schwann cells and macrophages. Incomplete regeneration of peripheral nerves, especially when a long defect is created, can lead to neuropathic changes. Application of transplantation procedures in regenerative medicine developed new methods to accelerate regeneration of injured peripheral nerves or restore the functions of interrupted axons by using isogenic epineurium. Epineural sheath was successfully applied for sciatic nerve regeneration in a rat model (Siemionow et al., 2010). Epineurium was also found as a natural conduit filled with bone marrow stromal cells to bridge gaps in peripheral nerves after injury in an experimental model, and it was proven that nerve regeneration was comparable to nerve autografts (Siemionow et al., 2011). These results demonstrated that isogenic epineurium is a promising natural material for application for peripheral nerve repair after peripheral nerve injury. However, in the clinical scenario, there is an insufficient amount of nerve material available for harvest and for autograft transplantation without affecting the motor and sensory function of the patient. From this point of view, it is important to find the opportunity for nerve acquisition for clinical use, without the risk of an influence on the nerve function of the donor. Moreover, it is important to complete the knowledge about biological properties of the epineurium for potential application for allogeneic recipients. Previous data provided limited information about biological properties of the human epineurium. Therefore the aim of this study was to assess immunoreactivity, proangiogenic and neurogenic features of epineurium isolated from peripheral nerves taken from deceased organ donors and from limbs amputated due to critical limb ischemia as an alternative, for autologous nerves, source of tissues.

Materials and methods

Tissue samples

Peripheral nerves were collected from 10 deceased organ donors taken from the ilioinguinal nerves (19 samples), and from 7 patients taken from sciatic nerves (12 samples), from limbs amputated above knee level due to critical limb ischemia associated with diabetes mellitus. Procedures were performed with local Bioethics Committee approval (No. KB - 479/2015). The mean age of patients in the group of organ donors was 36.7 years (range 23-67) (gender 3F/7M), and in the group of amputated limbs it was 66 years (range 54-86), (gender 2F/5M). In the case of organ donors, ilioinguinal nerve samples were collected immediately after kidney harvest for transplantation. Nerves gathered from amputated limbs were isolated immediately after the surgical procedure of amputation was completed. Immediately after nerve dissection, tissues were placed in 0.85% sodium chloride. Subsequently nerve sections were divided and one part was fixed in 4% formalin, whereas the other part was surgically prepared and an empty epineural sheath was created after removal of nerve fascicles using the pull out technique as previously described (Siemionow et al., 2011). Specimens were moved through all the steps - processing, paraffin embedding and sectioning - to obtain microscope slides with nerve fascicles in cross and longitudinal sections and the epineural sheath alone on each slide. Before immunocytochemical analysis nerves and epineurium architecture were assessed by routine hematoxylin and eosin (H+E) staining.

Immunostaining

Prior to immunostaining slides were deparaffinized in a series of xylene and a gradient of ethanol (absolute, and 95%, and 70% ethanol, respectively) and rehydrated in deionized water. Heat-induced epitope retrieval was performed in a 97°C water bath for 20 minutes in one of two buffers - citrate buffer pH=6.0 or TRIS/EDTA buffer pH=9.0 -according to primary antibody specification. To assess epineurial immunoreactivity we evaluated expression of human leukocyte antigen class I (HLA class I, clone EMR8-5, Abcam) and class II (HLA class II, clone TAL 1B5, Santa Cruz Biotechnology) and presence of immunocompetent cells including T lymphocytes (CD3, clone F7.2.38, Dako; CD4, clone 1F6, Leica; CD8, clone C8/144B, Dako) and macrophages (CD68, clone PG-M1, Dako). Neurogenic properties were assessed by the presence of S-100 (clone S1-61, Santa Cruz Biotechnology), GFAP (polyclonal, Abcam) and laminin (clone 4C7, Dako). Proangiogenic markers were examined by staining for CD31 (Platelet Endothelial Cell Adhesion Molecule PECAM-1, clone JC/70A, Abcam) specific for endothelial cells, and for VEGF (Vascular Endothelial Growth Factor, clone EP1176Y, Abcam). Primary antibodies were incubated for 30 min. Between each step of immunostaining slides were washed in TRIS/NaCl buffer pH=7.6. Visualization was performed by using the Dako Envision G/2 System/AP kit detecting mouse and rabbit primary antibodies. Cell nuclei and other basophilic structures were counter-stained with hematoxylin. Slides were covered in mounting medium and analyzed under the light microscope and assessed according to the guidelines specific for each parameter.

Assessment of immunological properties (HLA class I and class II, CD3, CD4, CD8, CD68)

HLA staining was assessed for their expression separately for epineurium, vessel endothelium and mononuclear cells. In addition, staining of vessel endothelium was graded subjectively according to intensity of HLA class I and class II expression: no expression (-), weak (+), moderate (++) and strong (+++). Lymphocytes and macrophages were counted in five areas of a high power field (HPF) at magnification 400x, separately in epineurium and in nerve fascicles. Average number of cells in one HPF were assessed for CD3, CD4 and CD8 lymphocytes. Macrophages identified by CD68 antigen were evaluated according to a semiquantitative scale relative to physiological presence of these cells in tissues: cells not found (-), 1-10 cells (+), 11-20 cells (++), >20 cells (+++).

Assessment of neurogenic properties (GFAP, S-100, laminin)

Neural specific antigens used in this study were Ca^{2+} binding protein S-100 and GFAP, which is one of the intermediate filaments. Both of them are also present in other tissues, but the aim of this research was to confirm lack of expression of these markers in the epineurium. Laminin was added to this group because of its ability to promote axonal regeneration in peripheral nerve injuries by providing a scaffold for new axons. However, laminin is also used as a proangiogenic marker by its presence on vessel endothelial cells.

Assessment of vascularization (CD31, VEGF)

CD31, known as platelet endothelial cell adhesion molecule (PECAM-1), was applied in immunohistochemistry mainly to identify vessel density by expression on vessel endothelial cells. In each stained slide, vessels positioned in the epineurium were counted in five HPF at magnification 400x. To assess angiogenesis anti-VEGF antibody was used.

Statistics

Cell count (CD3, CD4, CD8), vessel density (by CD31) and angiogenesis (by VEGF) in the epineurium of both groups - from organ donors and from amputated limbs - were calculated as mean and standard deviation (SD) and were compared statistically with the non-parametric Mann-Whitney U-test for independent samples at a significance level of 0.05.

Results

Nerves and epineurium architecture

Staining with hematoxylin and eosin (H+E) was performed to reveal the histological structure of examined tissues. Histology of examined ilioinguinal nerve samples from organ donors confirmed normal architecture, as previously described (Sunderland, 1990). Nerve fascicles, composed of axons, Schwann cells and endoneurium, were surrounded by perineurium. A few nerve fascicles were bundled by the epineural sheath and formed a nerve. In the isolated epineurium blood vessels and single scattered mononuclear cells were present (Fig. 1). In the case of sciatic nerves from amputated limbs, impaired histological structure was observed. Blood vessels were dilated and often filled with mononuclear cells. The epineurium in this group was poorer and in the majority of samples (in 7 out of 12 samples) dominated by adipose tissue.

Immunological parameters

Expression of HLA class I antigens was observed on the vessel endothelium localized within the epineurium in all examined samples (Fig. 1). There were no significant differences between expression of HLA class I antigen on the vessel endothelium in epineurium taken from organ donors or from amputated limbs. In contrast, the expression of HLA class II antigens on the vessel endothelium in epineurium from sciatic nerves from amputated limbs was stronger compared to the vessel endothelium present in the epineurium obtained from organ donors. Semiquantitative analysis revealed that in the epineurium from ilioinguinal nerves, expression of HLA class II antigen on the vessel endothelium was absent or less abundant (not exceeding grade +) than in the group of amputated limb.

Mononuclear cells with expression of HLA class I antigens were found in perivascular localization in the epineurium taken from ilioinguinal nerves; however, these cells were more numerous (more than 6 cells in 5 HPF) in the epineurium taken from sciatic nerves from amputated limbs, as illustrated in the epineurium surrounding nerve fascicles (Fig. 1).

Results summarized in Fig. 2 demonstrate differences in the number (statistically not significant) of CD3+ T lymphocytes in the epineurium between the two groups: from organ donors and from amputation (3.82±2.73 vs 4.34±4.59 respectively). The histomorphological image shows lymphocytes forming clusters in the tissues taken from sciatic nerves from amputated limbs, in contrast to organ donors' tissues, where CD3 lymphocytes were present occasionally in the epineurium and were localized in the vicinity of the vessels (Fig. 1). Comparing subpopulations of CD4 (T-helper) and CD8 (T-cytotoxic) T lymphocytes, the CD8+ cells were slightly more numerous in the epineurium of both groups from organ donors and from amputations. However, the number of both CD4 and CD8 lymphocytes was greater in the epineurium taken from peripheral nerves from amputated limbs (CD4= 3.13±2.45; CD8=3.57±3.28) than in the epineurium obtained from organ donors (CD4 =2.16±1.37; CD8= 2.35±1.57) (Figs. 1, 2).

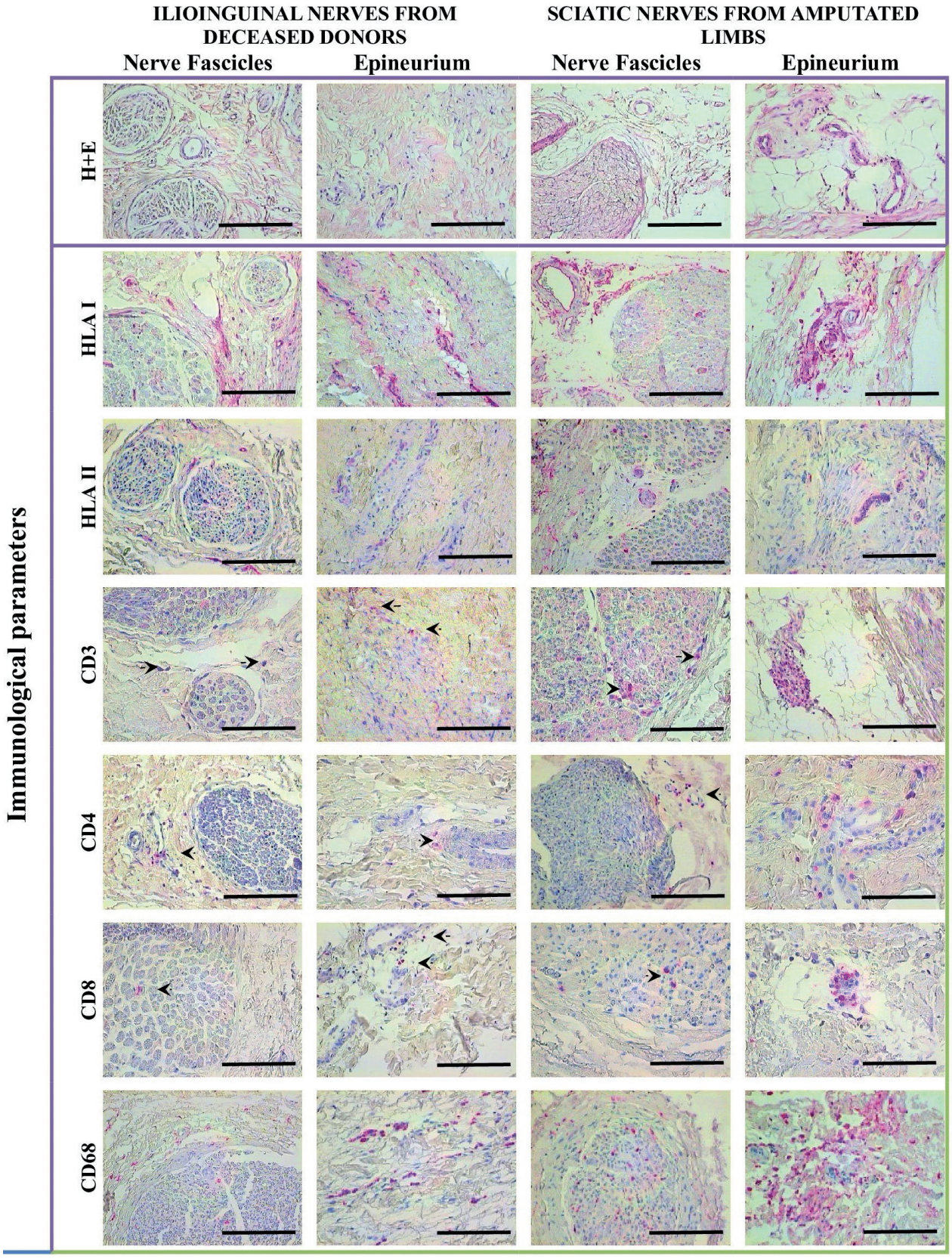

Fig. 1. Histological structure of examined tissues assessed by hematoxylin and eosin (H+E) staining, and immunological factors (HLA class I, HLA class II, CD3, CD4, CD8, CD68) in the intact nerves and in the isolated epineurium obtained from ilioinguinal nerves from deceased donors and from sciatic nerves from limbs amputated due to critical limb ischemia. Black arrowheads indicate positively stained cells. Scale bars: 200 μm

Macrophages, identified by CD68 antigen, were detected in the epineurium of both groups, but their number was greater (more often 11-20 cells/HPF) in the epineurium isolated from amputated limbs compared to epineurium isolated from ilioinguinal nerves from organ donors (less than 11 cells/HPF) (Figs. 1, 2).

Neurogenic properties of the epineurium

All nerve tissue samples showed high expression of S-100 protein in the majority of axons within nerve fascicles. In contrast, weak GFAP expression was detected on the axons only in one nerve tissue taken from an organ donor. Other tissue samples did not show any positive GFAP reaction on the axons. S-100 or GFAP was not present in the epineurium in both examined groups (Fig. 3).

In the peripheral nerve the expression of laminin supports nerve regeneration and angiogenesis. Normal expression of laminin was observed on the perineurium and on the endothelium of the blood vessels, with variable intensity between samples in the ilioinguinal nerves taken from organ donors. The perineurium also demonstrates diverse expression of laminin within both groups, but in nerves taken from amputated limbs, the perineurium surrounding nerve fascicles, with poor expression or without laminin expression, was more often visible than in the perineurium of ilioinguinal nerves from organ donors (Fig. 3).

Proangiogenic properties of the epineurium

Proangiogenic properties were analyzed by vessel density with CD31 and VEGF expression. Vessel density with CD31 expression was higher in epineurium from peripheral nerves of organ donors (4.60±1.27) compared to epineurium from amputated limbs (3.25±1.02); p=0.0053. The number of vessels expressing VEGF was significantly higher in epineurium from peripheral nerves of organ donors compared to those from sciatic nerves from amputated limbs (2.73±0.98 vs 0.93±0.38 respectively; p<0.0001 - Mann-Whitney U-test (Fig. 3, Fig. 4).

Discussion

Recent studies on the regenerative potential of different material, to prevent or restore peripheral nerve injury, have focused on biologic and synthetic material which would be clinically applicable in regenerative medicine (Siemionow et al., 2010; Johnson et al., 2013; Yan et al., 2014). This material should be easy accessible, biodegradable, with low toxicity and low antigenicity. It is important to understand the biological properties of epineurium when determining the options for nerve repair after injury. The aim of this study was to compare biological properties of epineurium from different sources to understand which biological factors may influence nerve regeneration without induction of

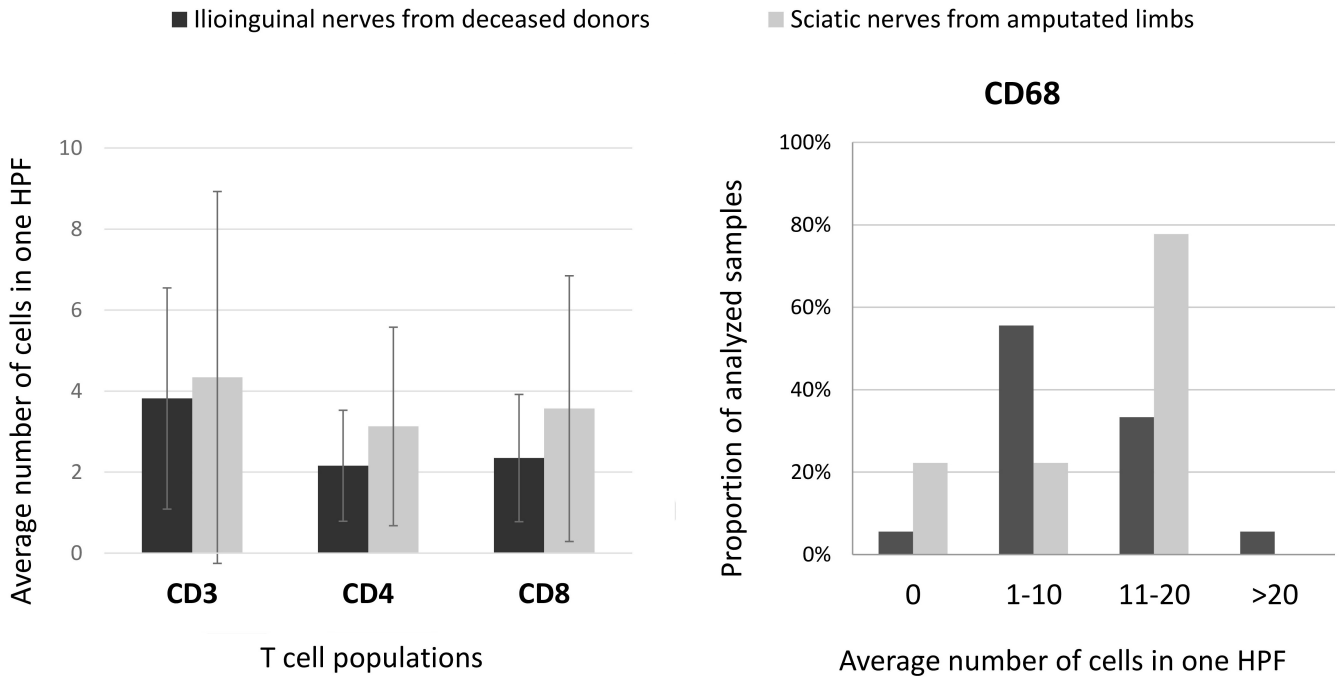

Fig. 2. Quantitative analysis of immunocompetent T cells (CD3) including subpopulation of helper CD4+ and cytotoxic CD8+ T lymphocytes and semiquantitative analysis of macrophages (CD68) in the epineurium from ilioinguinal nerves from deceased donors and from sciatic nerves from amputated limbs.

undesirable effects eg. inflammation. The epineurium isolated from the peripheral nerve samples of organ donors comes from individuals who were in good condition, in contrast to the second group with critical limb ischemia, where the inflammation is an integral feature of these cases and amputation is a terminal method of the treatment. This difference is evident by lymphocyte infiltrations which are more abundant and often form inflammatory aggregates in the epineurium originating from amputated limbs but not in the epineurium from ilioinguinal nerves from organ donors. Moreover, in epineurium from amputated limbs, the presence of clusters with prevalence of CD8 cytotoxic T lymphocytes and single CD4 lymphocytes, as well as

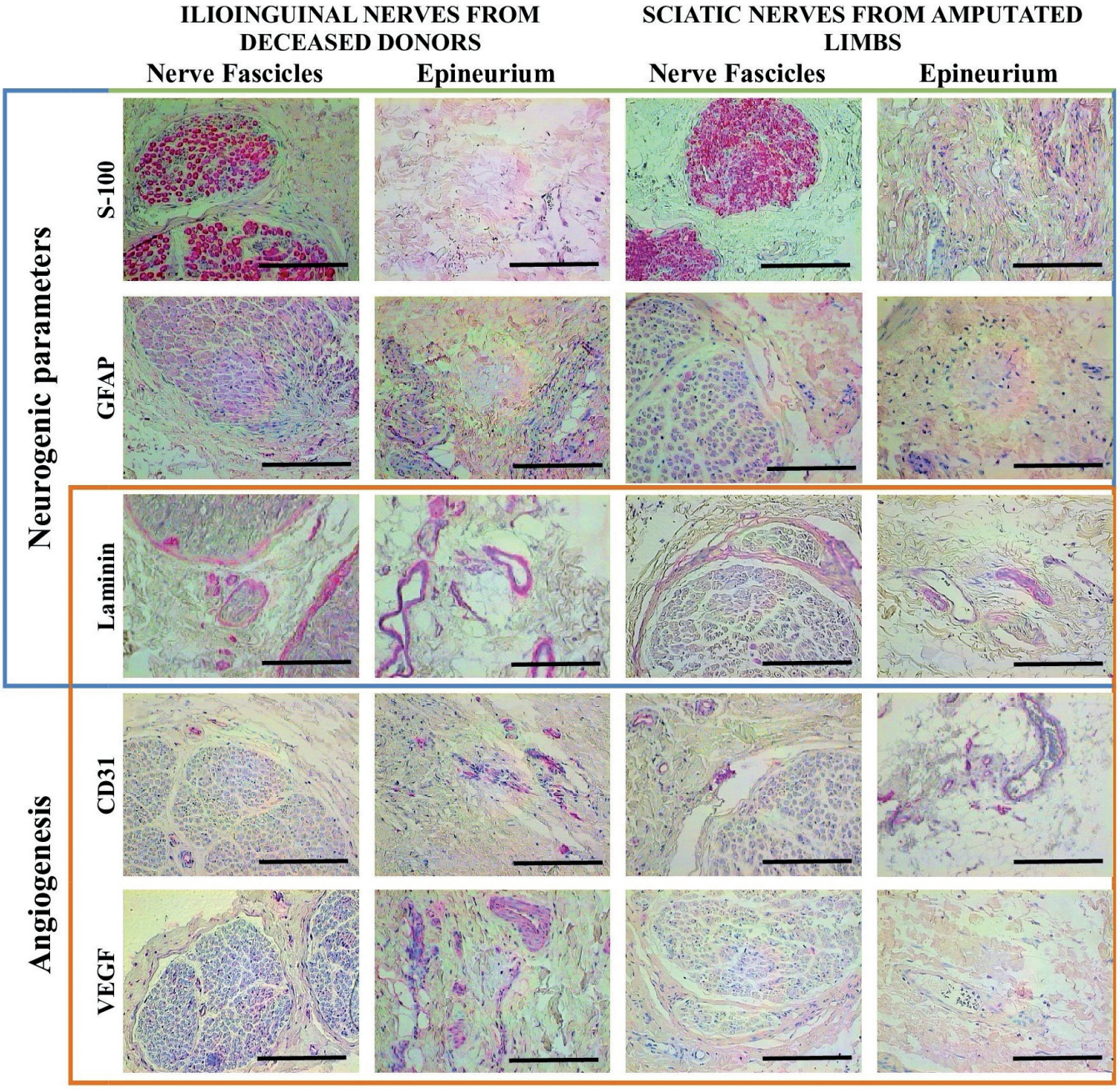

Fig. 3. Neurogenic and proangiogenic factors in the intact nerves and in the epineurium of ilioinguinal nerves obtained from deceased donors and from sciatic nerves from amputated limbs. Note that laminin overlaps both proangiogenic and neurogenic properties in the peripheral nerves. Scale bars: 200 μm.

HLA class II positive cells, confirmed the active inflammatory state. Additionally, lymphocytes have a propensity to stay in the epineurium, which is evident from the considerably fewer T cells within nerve fascicles in both examined groups. It may be a consequence of differences in vascularization, because blood vessels cross the perineurium-epineurium junction, whereas the lymphoid network is restricted to the epineurium (Topp and Boyd, 2006).

The presence of HLA class I and class II antigen expression is very important from the transplantation viewpoint. Any transplantation across the HLA barrier may induce an immune response and generate inflammation; however, the severity of the immune response is associated with the presence of immunocompetent cells within the transplanted tissues (Klimczak and Siemionow, 2010). The epineurium did not demonstrate positive reactions for HLA class I and/or class II antigens in either examined group. However, within the epineurium numerous vessels showed strong HLA class I expression, without significant differences between samples from organ donors and from amputations. Expression of HLA class I antigen on the vessel endothelium reflects the physiological state, as this protein is present on the surface of all nucleated cells of the body. The mononuclear cells expressing HLA class II antigens seem to involve mainly perivascular lymphocytes, but there were also found single macrophages and single Schwann cells in nerve fascicles. Our observations are consistent with previous reports assessing HLA class II expression in normal and pathological nerves (Scarpini et al., 1990). An increased number of HLA class II positive cells, which act as antigen-presenting cells, in the epineurium from limbs amputated due to critical limb ischemia may function as a chemoattractant for macrophage infiltration. We did not observe the presence of activated cells in the epineurium from ilioinguinal nerves from organ donors, which may have a beneficial effect on low epineurial immunoreactivity.

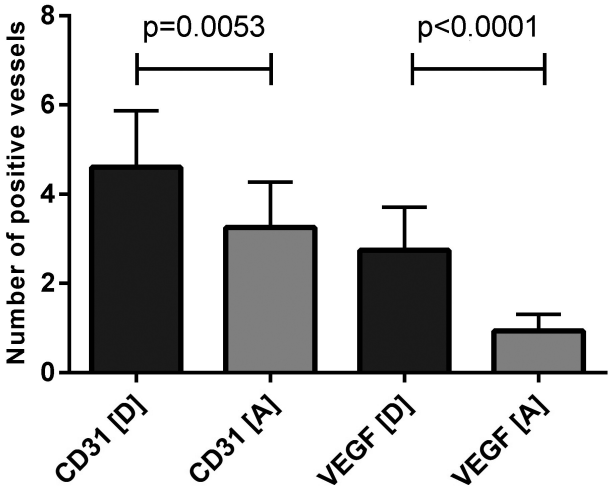

Fig. 4. Vessel density (mean ± SD) with positive staining for CD31 and VEGF in the epineurium of ilioinguinal nerves obtained from deceased donors [D] compared to epineurium from sciatic nerves from amputated limbs [A]. P-value - Mann-Whitney U-test.

Macrophages are the most important immune cells, playing key roles in peripheral nerve injury and repair. Tissue-resident macrophages are constitutively deployed throughout the nerve fiber and are responsible for removing damaged cells, myelin debris or old elastin, collagen and laminin fibrils (Kiefer et al., 2001). Macrophages were distributed in the epineurium in all investigated samples, but they were more numerous in the epineurium from nerves from amputated limbs, and this suggests their role in peripheral nerve injury in the course of critical limb ischemia. However, it is evident that after peripheral nerve injury a large number of macrophages are accumulated at the injury sites, where they not only contribute to nerve deterioration, but also are triggered by the local microenvironment and polarized to an anti-inflammatory phenotype (M2), and promote axonal regeneration (Chen et al., 2015). However, immunological features of the epineurium, isolated from peripheral nerves of amputated limbs, represented by an increased number of macrophages and accumulation of CD8+ T lymphocytes, may induce an immune response, making this material unsuccessful in allogeneic conditions. In contrast, moderate or low numbers of tissue-resident macrophages in the epineurium from deceased organ donors have a more beneficial effect on the biologic properties of this epineurium, especially when the anti-inflammatory phenotype of macrophages (M2), which contribute to homeostatic maintenance of peripheral nerve regeneration, predominates over the pro-inflammatory phenotype (M1) of macrophages, as reported by (Chen et al., 2015).

As mentioned above, this study did not reveal any significant differences between the two groups for expression of neurotrophic factors S-100 and GFAP. S-100 protein is normally present on Schwann cells and myelin sheaths in axons (Nakajima et al., 1982). In the case of our research we confirmed expression of S-100 on myelin sheaths and lack of positive staining in the epineurium. Lack of GFAP expression in almost all samples in both groups suggests no immunoreactivity in normal peripheral nerves, and it correlates with previous data (Memoli et al., 1984).

Laminin as a basic component of basement membrane, including epithelial, endothelial, smooth and striated muscle and peripheral nerve, appears predominantly in blood vessels (Durbeej, 2010). In addition, in the peripheral nerves laminin plays a supportive role in maintaining the perineural barrier, created by the tight junctions between perineural cells, which is involved in maintenance of an appropriate endoneurial environment and provides a scaffold for

axons. In the peripheral nerves laminin shows overlapping proangiogenic and regenerative potential (Allodi et al., 2012; Gonzalez-Perez et al., 2013; Yousif et al., 2013). Our studies confirmed laminin expression on the perineurium surrounding nerve fascicles and on the vessel endothelial cells in the epineurium of ilioinguinal nerves, and this finding proved the proangiogenic potential of epineurium isolated from organ donors. In contrast, proangiogenic properties, associated with laminin expression, were not established on the epineurium taken from sciatic nerves from amputated limbs. This may be a repercussion of damage caused by inflammation or the random result of laminin composition, which may change along peripheral nerves (Jaakkola et al., 1993). Our studies clearly documented that laminin expression is stronger in vessel endothelium obtained from a deceased donor compared to that from amputated limb. Thus, the presence of laminin on the vessel endothelium in the epineurium is more closely associated with angiogenesis than with axonal regeneration, because for axonal regeneration laminin expressed on the perineurium has an influence (Hill and Williams, 2002).

To date there are reports indicating that ischemia or chronic inflammation, of blood as well as lymphatic vessels, may lead to an increase of epineurial vascularization (Llewelyn et al., 1998). In contrast, our observations showed a significant decrease of vessel density, identified with anti-CD31 antibody, in the epineurium isolated from sciatic nerves from limbs amputated due to critical limb ischemia. Moreover, these vessels were biologically inactive, as they did not express the proangiogenic factor VEGF, compared to the vessel endothelium of epineurium isolated from organ donors. VEGF is a signal protein involved in the creation of a network of blood vessels during embryogenesis or in tissues after injury. Its activity is mostly specific for vessel endothelium but can also appear on other cells such as monocytes or macrophages (Ferrara and Davis-Smyth, 1997), and is also considered as growth-promoting activity on cells in the nervous system (Sondell et al., 1999a). The expression of the proangiogenic factor VEGF on vessel endothelium was greater in epineurium obtained from nerves from deceased organ donors, and this suggests that proangiogenic abilities of this epineurium are still preserved. The difference in vessel density in both examined groups may be due to progressive destruction of the vessel endothelium in amputated limbs. Additionally, epineurial sheaths in this group of samples were degenerated and dominated by adipose tissue, which may also play an important role in the diminished ability of neovascularization.

Interestingly, previous studies proved that besides promoting vascularization, VEGF triggers growth of neurons and glial cells, and can support their survival and outgrowth (Sondell et al., 1999b; Carmeliet and Storkebaum, 2002). Thus, proangiogenic properties of the epineurium of ilioinguinal nerves from organ donors, proved by expression of VEGF on the vessel endothelium, make this biologic material more valuable for clinical application for peripheral nerve regeneration compared to that from amputated ischemic limbs.

Studies on regenerative potential of different materials for peripheral nerve restoration are focusing on biologic material which would be clinically applicable in regenerative medicine. Epineurium from ilioinguinal nerves from organ donors has low immunoreactive properties, which is especially demonstrated by low expression of HLA class I and lack of HLA class II antigens, and the physiological number of macrophages, which constantly patrol peripheral nerves. This analysis of the immunologic, neurogenic and proangiogenic properties of the human epineurium revealed that material from organ donors constitutes a safer tool and may serve as biologic material in transplant and regenerative medicine in allogeneic conditions.

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Conflict of interests. The authors declare that there is no conflict of interests regarding the publication of this paper.

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