Expression of vascular endothelial growth factor receptors coincide with blood vessel in-growth and reactive bone remodelling in experimental intervertebral disc degeneration

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Abstract

Objective

To analyze immunohistochemically the localization of the VEGF receptors in experimental intervertebral disc degeneration tissues in a pig model.

Material and Methods

In six domestic pigs, the cranial bony endplate of the L4 vertebra were perforated into the nucleus pulposus. Three months postoperatively, the animals were sacrificed and the experimental and control vertebrae, complete with intervertebral discs, were excised and subjected for immunohistochemical staining of vascular endothelial growth factor receptors (VEGFR) along with VEGF - A, -C, -D and blood and lymphatic vessel markers vWF and LYVE-1.

Results

The results of immunohistochemical analysis of experimental samples showed VEGFR-1 (Flt-1) expression in intervertebral disc and all paradiscal tissues studied. In control samples expression of VEGFR-1 was lower and absent in the intervertebral discs. Comparatively less of VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) than VEGFR-1was found in degenerated intervertebral discs and paradiscal tissues. In contiguous control intervertebral discs and control paradiscal tissues VEGFR-2 and-3 receptors were expressed to a lower extent than in experimental tissues or were even totally absent. Also growth factors VEGF-A, -C, -D, as well as von Willebrand factor and to a much lower extent LYVE-1 were differently expressed in experimental and control intervertebral discs.

Conclusion

In experimental intervertebral disc degeneration, VEGF receptors were expressed in the damaged disc and paradiscal tissues. In the same tissues, VEGF-A, -C, and -D, signs of blood and lymphatic vessel in-growth and reactive/adaptive vertebral bone remodelling were found.

Key words

VEGF receptors, intervertebral disc degeneration, experiment.

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Introduction

Intervertebral discs connect the vertebrae of the spinal column together. They provide flexibility and support, and act as shock absorbers. There are great differences in the statics of the spine between different mammalian species but some basic biological processes related to repair are comparable in all. The intervertebral disc consists of two distinct parts, the inner nucleus pulposus and the outer annulus fibrosus, and it is the largest avascular tissue in the body. Maintenance of an adequate nutrient supply has long been regarded as crucial for preventing disc degeneration (1, 2). Essential nutrients such as oxygen and glucose and substrates for matrix production, such as amino acids and sulphates, are supplied to the disc from the blood vessels at the disc margins (3-5). These nutrients then move through the dense extracellular matrix of the disc to the cells, which can also be found in the centre of the nucleus pulposus, which, in an adult human lumbar disc, may be at a distance of 7 to 8 mm from the nearest blood supply. Oxygen and carbon dioxide tension in the tissues is dependent on the network of patent blood vessels and influence many local tissue properties. Disorders that affect the blood supply to the vertebral body, such as atherosclerosis of the abdominal aorta, are associated with disc degeneration and back pain (6, 7). Apart from disturbances of the blood supply, nutrients may not reach the disc cells if there is sclerosis of the subchondral bone or if the cartilaginous endplate calcifies (8). Small, uncharged solutes (oxygen, amino acids, water) diffuse across the endplate readily, but anions are partially excluded and the extent of steric exclusion of larger solutes increase with their molecular weight and is greater for linear than globular molecules (8).

Further evidence for disturbances of transport in degenerate discs are the high levels of lactic acid and low pH values measured in degenerate discs (9, 10). Direct measurements have shown that transport of magnetic resonance imaging (MRI) contrast media into the disc was inhibited in early degeneration (11), whereas in more degenerate discs, there were noticeable disturbances of the endplate region. Only low concentrations of large molecules (*e.g.*, growth factors, protease inhibitors) can enter the disc; even glucose is restricted to some extent (12). Charge effects, however, enhance the penetration of small cations such as sodium in direct proportion to the proteoglycan concentration, whereas anions such as sulphate and chloride are partially excluded (12). The periphery of the annulus fibrosus appears to be comparatively well vascularized, more so in children than in adults (13). In infants, blood vessels penetrate into the annulus fibrosus but these disappear by late childhood apart from some small capillaries, accompanied by lymph vessels, in the outer 1-2 mm of intervertebral disc (13, 14).

Angiogenesis is mediated by angiogenic factors (*e.g.*, VEGF) and regulated by hypoxia, inflammatory cytokines and mechanical load (15). Histological examination of the anterolateral part of the annulus fibrosus showed that the vascularity of the annulus fibrosus increased significantly with degeneration of the disc, and most of the arteries had a vertical orientation. There are not many articles concerning VEGF participation in intervertebral disc degeneration, and little data on its receptors in this process (16). During adaptive tissue repair following intervertebral disc degeneration blood vessel proliferation can be seen as an attempt of unspecific regeneration, which however does not reproduce intervertebral disc structure and function.

VEGF participates in bone remodelling (17-28) but even less information is available on VEGF and its receptors in reactive/adaptive bone processes in intervertebral disc degeneration.

The aim of this study was to analyze the expression of VEGF and its receptors in experimental lumbar intervertebral disc injury as potential factors responsible for new vessel formation and vertebral bone remodelling. The hypothesis was that loss of the normal biomechanics of the disc as a result of the injury activates compensatory angiogenesis and ectopic growth of blood vessels and spinal bone remodelling.

Material and methods

The experimental procedure has already been described (29). In short, six domestic pigs, initially 4-5 months of age and weighing 50-60 kg, were used in the study. After the 3-month post injury period, the pigs were sacrificed. Ethical approval for the use of animals in this study was granted by the Animal Research Ethics Committee of Göteborg.

Endplate and intervertebral disc injury surgical procedures

Each animal was first sedated by an intramuscular injection of Ketalar (ketamine, animal was anesthetized with intravenous injections of Hypnodil (methomidate chloride, 3-5 mg/kg of body weight; AB Leo, Helsingborg, Sweden) and Stresnil (azaperone, 0.1 mg/kg of body weight; Janssen-Celag, Sollentuna, Sweden). With the animal lying on its right side, the L3-L4 disc was exposed using a left retroperitoneal approach. The cranial endplate of the L4 vertebra was perforated using a 3.5-mm drill bit inserted from the lateral cortex at mid-height. Angulated at 45° so as to reach the central part of the endplate, a single hole was drilled into the nucleus pulposus and then withdrawn. Postoperatively, the animals recuperated in an animal facility for a period of 3 months, where they were monitored daily.

After the animal was sacrificed, the spine was excised, frozen, and later underwent histological, and immunohistochemical examinations of the degenerated and adjacent intervertebral discs as controls. To obtain a series of slices parallel to the edge of the annulus, a full-thickness rectangular strip approximately 20 mm in lateral width was cut from the anterior to the posterior part of the disc. This was done for the degenerated and control discs. Histological sections were cut in perpendicular orientation from this rectangular specimen containing the remnants of nucleus pulposus and annulus fibrosus (or in the control sample the disc itself) together with cartilaginous and bony vertebral endplates and some vertebral body bone marrow.

Histological analysis

Samples were fixed in 4% neutral formaldehyde and decalcified. Tissue samples were then dehydrated and embedded in paraplast. From the resulting blocks, 3-4-mm thick sections were cut and mounted on L-polylysine-coated slides for routine staining with haematoxylin and eosin and subsequent immunohistochemistry.

Immunohistochemical analysis

Anti-human VEGF receptors and VEGF antibodies were tested in pilot studies for their reactivity in porcine tissues using lung from porcine enzootic pneumonia caused by *Mycoplasma hyopneumoniae* and gut from proliferative enteropathy caused by *Lawsonia intercellulare*. Fixation used in these pilot studies was the same as was used in the actual experiments. Only those antibodies, which showed clear interspecies cross-reactivity were used in the experiments described below. These were anti-VEGFR-1, -2, and -3, VEGF-A, -C, -D, von Willebrand factor (vWF) and LYVE-1 antibodies.

Primary antibody used:

1) Rabbit polyclonal anti VEGFR-1 affinity purified IgG mapping epitope at the C-terminus of human origin (Fitzgerald Industries International, formerly Research Diagnostics Inc., Concord, MA, USA), 2.9 mg/ml; 2) Rabbit polyclonal anti human VEGFR-2 affinity purified (Fitzgerald Industries International, formerly Research Diagnostics Inc., Concord, MA, USA), 4 mg/ml; 3) Rabbit polyclonal anti-human VEGFR-3 antibody (Fitzgerald Industries International, formerly Research Diagnostics Inc., Concord, MA,USA), 1 mg/ml; 4) Rabbit polyclonal VEGF-A (Fitzgerald Industries International, formerly Research Diagnostics Inc., Concord, MA, USA), 2mg/ml; 5) Goat polyclonal N-terminal VEGF-C (Santa Cruz Biotechnology, Santa Cruz, CA), 2 mg/ml; 6) Goat polyclonal N-terminal VEGF-D (Santa Cruz Biotechnology, Santa Cruz, CA), 2 mg/ml; 7) Rabbit polyclonal von Willebrand Factor (DAKO A/S, Glostrup, Denmark) 1:400 dilution; 8) Rabbit anti mouse polyclonal LYVE-1 (a gift from Kari Alitalo, Biomedicum Helsinki, Finland) 1:200 dilution.

Paraffin sections 5-mm thick were mounted on DAKO capillary slides (TechMate, DAKO), deparaffinized in xylene, and rehydrated in graded ethanol series and 10 mM phosphate-buffered, 140mM saline, pH 7.4 (PBS). For antigen retrieval, VEGFR-1, -2 and -3 were treated in 4 mg 1 ml pepsin (Merck, Darmstadt, Germany) solution for 45 minutes at +37°C, the rest of the slides were placed into the buffer for antigen retrieval for the use with TechMate Instruments (DAKO) and microwaved in a microwave processing labstation (MicroMED T/T Mega Histoprocessing Labstation, Milestone Inc, Atlanta, USA) for 10 minutes at 98° C according to the manufacturer's program, then kept at room temperature for 30 min, washed in PBS, and stained automatically with the following protocol: 1) the primary antibody for 1 h; 2) the secondary biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG antibodies for 30 min; 3) peroxidase block for 30 min; 4) peroxidase-conjugated streptavidin three times for 3 min; 5) HRP substrate buffer; and finally 6) substrate working solution containing 3,3'-diaminobenzidine tetrachloride (ChemMate Detection Kit) for 5 min. Between each step, the sections were washed with DAKO ChemMate washing buffers three times and dried in absorbent pads. Replacement of the primary antibodies with normal rabbit IgG or goat IgG diluted with DAKO ChemMate antibody diluent was used as negative control. After staining, the sections were removed from the machine, counterstained with haematoxylin or left without counterstaining, washed, dehydrated in ethanol series, cleared in xylene, and mounted in synthetic mounting medium (Diatex, Beckers Industrifäg, Märsta, Sweden).

Semiquantitative microscopic assessment of immunohistochemical staining (10 high power fields per animal for each staining) was performed under ×400 magnification using four grades: "–" = no immunoreactivity; "+" = only a few immunoreactive profiles; "++" = some immunoreactive profiles; "+++" = many immunoreactive profiles. In this work the "profile" means cell, cell processes and occasionally small extracellular matrix aggregate. Two histopathologists evaluated the results independently using a predefined and very simple scoring sys-

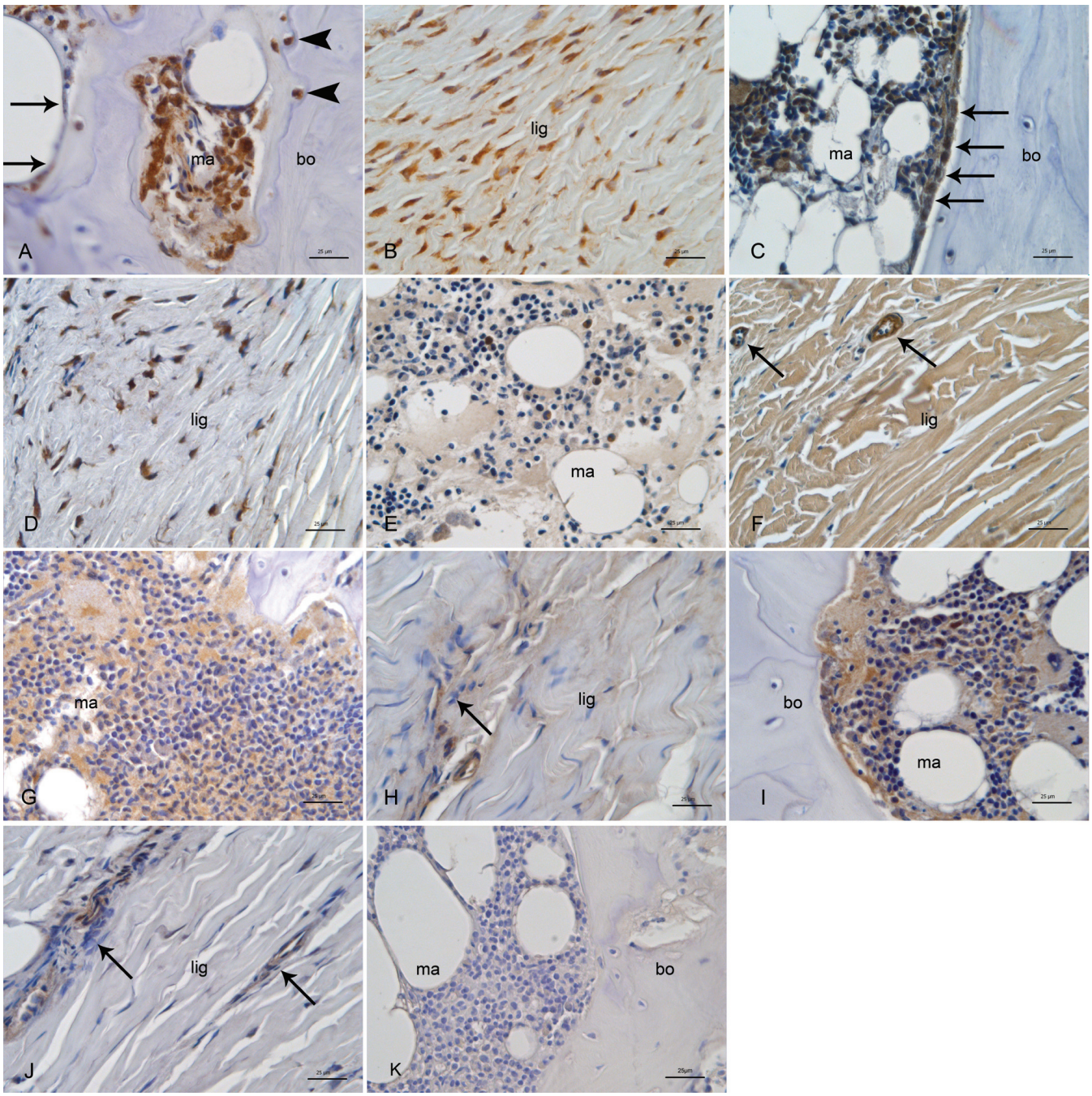

Fig. 1. Expression of three VEGF receptors in experimental intervertebral disc degeneration in swine. VEGFR-1 (**A** and **B** – experimental samples, **C** and **D** – control samples), VEGFR-2 (**E** and **F** – experimental samples), VEGFR-3 (**G** and **H** – experimental samples, I and J-control samples) and rabbit IgG as a negative staining control (**K**). **A** – VEGFR-1 in bone marrow and bone (some osteocytes are indicated with arrow heads and osteoblasts with arrows); **B** – VEGFR-1 in spinal ligament tenocytes; **C** – VEGFR-1 in bone marrow and bone (some osteoblasts are indicated with arrows); **D** – VEGFR-1 in spine ligament; **E** – VEGFR-2 in bone marrow; **F** – VEGFR-2 in spinal ligament (some blood vessels are indicated with arrows); **G** and **I**-VEGFR-3 in bone marrow cells; **H** and **J** – VEGFR-3 in spinal ligament (some blood vessels are indicated with arrows), **K** – negative staining control. Immunoperoxidase staining. Counterstained with haematoxylin. bo: bone; lig: spinal ligament; ma: bone marrow.

tem leading to very similar readouts and followed by a consensus session where the few and slight discrepancies were discussed. The results of histopathological evaluation obtained by routine staining with hematoxylin and eosin are presented in narrative form.

Results

Histopathological findings

The original trauma traces of experimental drill-made defects were not found. Histological analysis of the tissues stained with haematoxylin and eosin revealed that the majority of samples displayed signs of intervertebral disc degeneration in all experimental intervertebral discs studied. All samples showed signs of degeneration characterized by disorganization of lamellar structure of the annulus fibrosus, formation of fissures, cystic or myxomatous change,

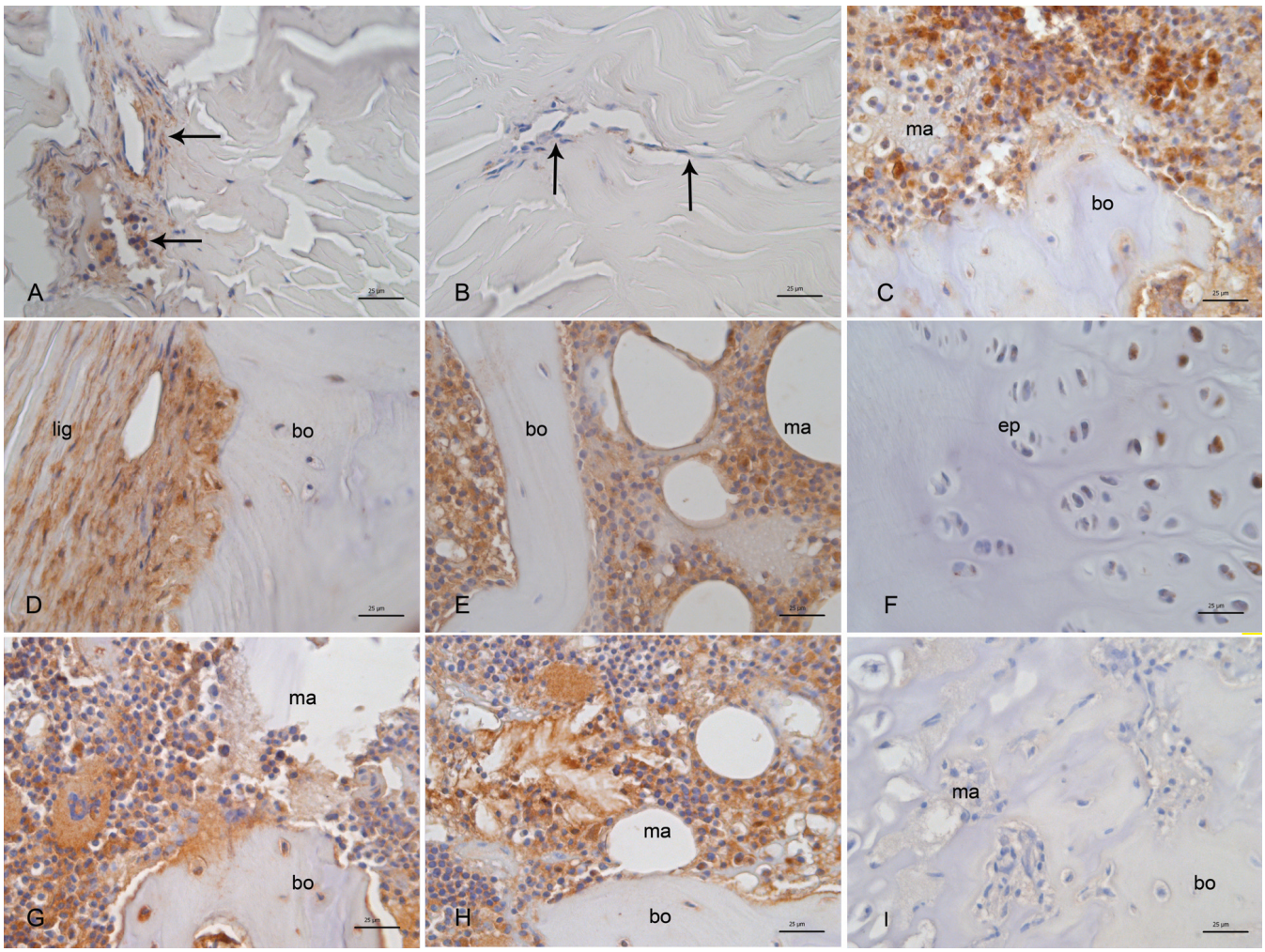

Fig. 2. Expression of VEGF in experimental intervertebral disc degeneration in swine. VEGF-A (**A** – experimental, and **B** – control sample), VEGF-C (**C** and **D** – experimental, **E** and **F**– control samples), VEGF-D (**G** – experimental, **H** – control samples). **A** and **B** – VEGF-A in annulus fibrosus (some blood vessels are indicated with arrows), **C** and **E** – VEGF-C in bone marrow, **D** – in spinal ligament, **F** – in endplate. **G** and **H** – VEGF-D in bone marrow. **I** – negative staining control. Immunoperoxidase staining. Counterstained with haematoxylin.
bo: bone; ep: endplate; lig: spinal ligament; ma: bone marrow.

cloning of chondrocyte-like cells and clefts formation within the cartilaginous endplate, and/or cartilaginous metaplasia or granular degeneration of the nucleus pulposus. Swelling of annular fibres and vacuole formation with or without structureless content, were frequently found in the changed annulus fibrosus. Vascular invasion and fibrochondrocyte-like cells were observed in outer part of annulus fibrosus and near the cartilaginous endplate.

Vertebrae disclosed osteophyte formation as well as fibrosis of the bone marrow and/or loss of predominantly longitudinal bone trabeculae orientation, mostly just beneath of endplate. Superficial bone erosion immediately beneath the periosteum was frequently seen in experimental samples, but not in control samples. Spinal ligaments also partially changed their normally strict orientation and package of fibres. In disorganized compact connective tissue, new in-grown blood vessels were seen.

In control samples, the annulus fibrosus either maintained its dense lamellar structure of collagen fibres or the lamellar structure was slightly disorganized. The nucleus pulposus underwent different degrees of fibrosing, but no granular degeneration was found.

Immunohistochemical findings

VEGF receptors

The aggregated data summarizing the findings of VEGF receptors expression in experimental intervertebral disc degeneration are shown in Table I.

Generally, the highest degree of expression in lumbar vertebral tissues was found for VEGFR -1 (Table I). In experimental samples many VEGFR-1 positively stained cells were found in bone marrow, mostly hematopoietic and sporadically in stromal cells (Fig. 1, panel A), periosteum, osteoblasts, osteocytes, in annulus fibrosus and spinal ligaments (Fig. 1, panel B). Degenerated nucleus pulposus in experimental samples contained VEGFR-1 positive cells. In control samples VEGFR-1 was expressed to a lower extent in periosteum, osteoblasts, bone marrow and spinal ligament tenocytes (Fig. 1, panels C and D). There was no VEGFR-1 staining in

Table I. Expression of VEGF receptors in experimental lumbar disc degeneration in the lesional sites and contiguous control sites.

VEGF receptor	Bone marrow		Vertebral bone		Vertebral endplate		Intervertebral disc		Spinal ligaments	
	Exp	Ctr	Exp	Ctr	Exp	Ctr	Exp	Ctr	Exp	Ctr
VEGFR-1	+++	++	++	+	+	+	++	–	+++	++
VEGFR-2	+	–	+	–	–	–	+	–	–	–
VEGFR-3	++	+	+	–	–	+	+	–	+	+

Exp: experimental samples; Ctr: control samples from contiguous surgically intact vertebrae. Score value: – = no positive profiles; + = a few positive profiles; ++ = some positive profiles; +++ = many positive profiles. In this work, the ”profile” means cell, cell processes and occasionally extracellular matrix staining.

intervertebral disc in control samples. VEGFR-2 was expressed only in experimental samples in periosteum, bone marrow, in some osteoblasts and in spinal ligament tenocytes (Fig. 1, panels E and F). No VEGFR-2 positive profiles were found in the contiguous control discs. VEGFR-3 staining was found in experimental samples, nevertheless to a lower extent than VEGFR-1 (Table I). VEGFR-3 positive cells were found in bone marrow (Fig. 1, panel G), but also in some osteocytes, osteoblasts, periosteal cells and spinal ligament tenocytes (Fig. 1, panel H). In control samples the expression of VEGFR-3 was similar or lower than in their experimental counterparts (Fig. 1, panels I and J), except in endplate, in which area this receptor in experimental samples was absent.

All negative staining controls using normal rabbit IgG were negative (Fig. 1, panel K), which confirms that VEGF receptor staining was true positive staining.

VEGF

In experimental samples expression of VEGF-A was found in some areas of annulus fibrosus annulocytes, but also in some blood vessels (Fig. 2, panel A), in some spinal ligament tenocytes as well as in some osteocytes and in cartilaginous endplate chondroblasts. Considerable numbers of positively stained cells were found among bone marrow cells, where high immunoreactivity was seen in numerous osteoclasts, megakaryocytes and hematopoietic cells. In contiguous control discs (Fig. 2, panel B), VEGF-A localization and degree of expression were comparable to those of experimental samples, or were slightly fewer and less intensively staining.

Immunohistochemical staining for the lymphangiogenic growth factor VEGF-C in experimental (Fig. 2, panels C and D) and control samples (Fig. 2, panels E and F) was more evident than angiogenic VEGF-A. It was prominently localized in bone marrow hematopoietic cells, some spinal ligament tenocytes, sporadic annulus fibrosus annulocytes, in some endplate chondrocytes and sporadically also in some osteocytes.

Considerably higher expression of VEGF-D, growth factor also mainly related to lymphangiogenesis, was found both in experimental (Fig. 2, panel G) and adjacent control vertebrae (Fig. 2, panel H), in numerous bone marrow cells and osteocytes. The intensity of staining was similar or slightly stronger in experimental samples than in contiguous control samples. Absence of brown colour in the negative staining control (Fig. 2, panel I) confirmed specificity of immunohistochemical reactions.

Von Willebrand factor

Von Willebrand factor immunoreactivity was found in bone marrow megakaryocytes, vascular endothelial cells and platelets, both in experimental and adjacent control vertebra samples (Fig. 3, panels A and D). In experimental samples vWF was found in degenerated experimental nucleus pulposus, containing newly formed blood vessels, occasionally with blood clots and extravasal leakage of vWF (Fig. 3, panel B). In annulus fibrosus and in spinal ligaments of experimental discs vWF-positive blood vessels were thin-walled and with rather narrow lumina (Fig. 3, panel C). In similar areas of control samples vWF-positive blood vessels generally had thicker walls and wider lumen diameters (Fig. 3, panel E). Negative staining control showed no vWF-expressing profiles (Fig. 3, panel F).

LYVE-1

In experimental samples LYVE-1 (Lymphatic Vessel Endothelial Receptor 1), a CD44 homolog, were found in some vessels in annulus fibrosus (Fig. 4, panel A) and in a similar localization in few vessels in contiguous control samples (Fig. 4, panel B). The degree of expression of LYVE-1 in control samples was lower than in experimental samples. Negative staining controls showed no brown staining (Fig. 4, panel C).

Discussion

In this experiment, acute surgical intervertebral disc injury in otherwise healthy pigs evidently caused the healing process in damaged vertebral bone, endplate and intervertebral disc. The operated animals were sacrificed three months after injury, when complete healing of all experimentally injured tissues was found. However, the healed tissues showed structural changes. The most striking changes were found in the normally avascular intervertebral disc. Stingy vascularization and dense fibrosis was found. Yet, this fibrosis, along with signs of granular degeneration and scarce blood vessels, was morphologically far from normal intervertebral disc. It seems that blood vessel in-growth into previously avascular areas was a prominent feature. The first step in the formation of most blood vessels is the production of an intricately branched vascular plexus (30-32). This plexus is subsequently pruned and remodelled and, in some cases, followed by coalescence to form larger

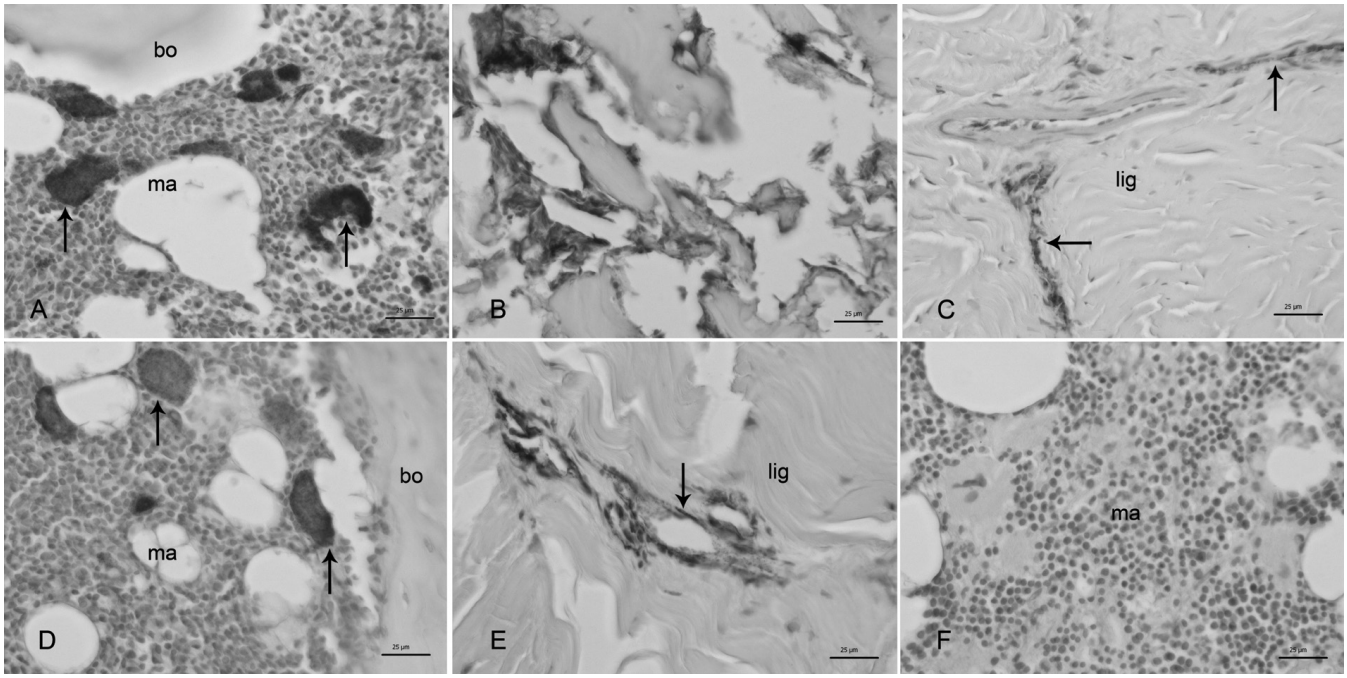

Fig. 3. Expression of von Willebrand factor in experimental intervertebral disc degeneration in swine. **A, B, C** – experimental samples, **D, E** – samples from adjacent control vertebrae. **A** and **D** – vWF in bone marrow (some megakaryocytes are indicated with arrows), **B** – vWF in degenerated intervertebral disc, **C** and **E** – in spinal ligaments (some blood vessels are indicated with arrows), **F** – negative staining control. Immunoperoxidase staining. Counterstained with haematoxylin.
bo: bone; lig: spinal ligament; ma: bone marrow.

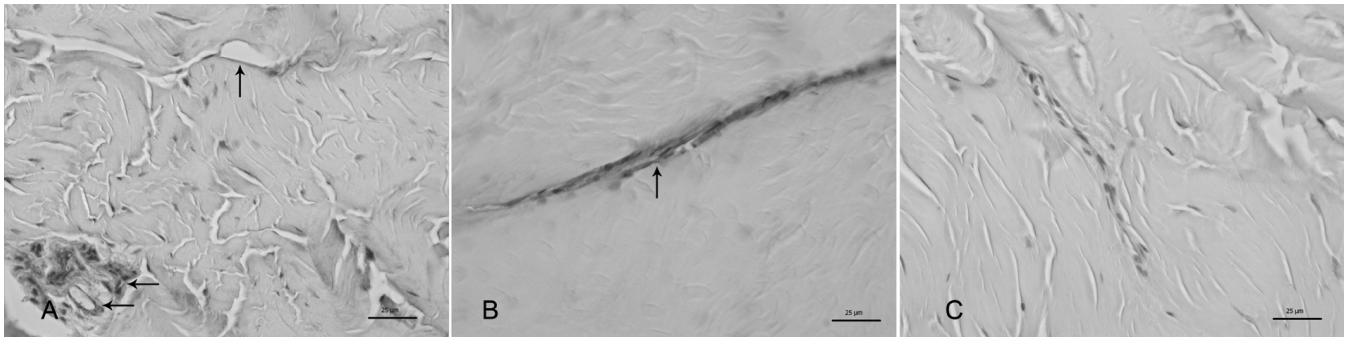

Fig. 4. Expression of lymphatic endothelial cell receptor LYVE-1 in experimental disc degeneration in swine. **A** – Experimental sample. LYVE-1 in vessels in annulus fibrosus (some vessels are indicated with arrows), **B** – LYVE-1 in the vessel in annulus fibrosus in control sample (indicated with arrow), **C** – negative staining control. Immunoperoxidase staining. Counterstained with hematoxylin.

vessels. The primary branched plexus is formed by several processes, including the initial assembly of vascular precursor cells called vasculogenesis, and a subsequent migration of endothelial cells from the parent vessel called sprouting angiogenesis. VEGF/VEGFR-2 interactions positively influence endothelial chemotaxis and proliferation (33, 34) and VEGF-A/VEGFR-1 ensure proper sprout formation during blood vessel formation (35). These two types of VEGFRs were increased in the experimental disc injury suggesting that repair in the damaged disc is coupled to up-regulation of these receptors which are important for endothelial cell chemotaxis, proliferation and sprout formation. That they really serve this function in this setting was not proven, but considering the simultaneous expression of these receptors and in-growth of blood vessels, as well as the role of VEGFR-1 and -2 in angiogenesis in general, this interpretation seems likely.

The angiogenic effects of VEGF are mediated by two transmembrane tyrosine kinase endothelial-specific receptors: VEGFR-1 (fms-like tyrosine kinase-1) and VEGFR-2 (kinase insert domain-containing receptor/fetal liver kinase-1 or KDR (36-38). Among the VEGF receptor (VEGFR) family members, VEGFR-3 also known as flt (fms-like tyrosine kinase)-4, is a receptor for VEGF-C and VEGF-D (39, 40). Expression of VEGFR-3 is developmentally regulated so that it is expressed on all of the embryonic endothelia, whereas its expression is largely restricted to the lymphatic endothelium in adult tissues (41). Thus, VEGFR-3 is a marker of lymphatic endothelial cells (42-45) and experimental studies have

revealed that activation of VEGFR-3 induces lymphangiogenesis (39). Lymphatic vessels often follow blood vessels. In present experiment VEGF-C, and VEGF-D were expressed in experimental and adjacent control vertebrae. The few tiny lymphatic vessel expressing the lymphatic endothelial marker LYVE-1 seen in annulus fibrosus suggest that this experimental porcine intervertebral disc degeneration model leads to up-regulation of lymphangiogenesis.

In addition to its role in angiogenesis, VEGF also participates in bone remodelling (17-28). It was, therefore, interesting to note that an experimental disc injury, likely to alter the biomechanics of the spine, was followed by histopathological changes in the bony and ligament structures of the spine. This was again associated with simultaneous changes of the three studied VEGFRs in these same locations so that the experimental samples clearly differed in this respect from the contiguous surgically intact vertebrae. Expression of VEGF receptors has been described in association of remodelling of some other bones (46, 47) so it might participate in such remodelling also in spine lesions although this might also be only coincidence. Accordingly, the main conclusion of the present work is that in an experimental intervertebral disc lesion VEGF receptor expression changes in the damaged disc and paradiscal tissues. At the same time blood and to some extent lymphatic vessel ingrowth and reactive/adaptive vertebral bone remodelling are found in these same tissues. It thus seems that a local injury and loss of the normal biomechanics of the disc as a result may activate ectopic growth of blood vessels and remodelling of the bony spine.

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