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# Detection of vascular endothelial growth factor (VEGF) in moderate osteoarthritis in a rabbit model

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ABSTRACT

*Introduction:* Vascular endothelial growth factor (VEGF) is detectable in later stages of human osteoarthritis (OA), but not in the healthy articular cartilage. Due to its capacity to increase matrix metalloproteinases and to decrease their inhibitors (tissue inhibitors of metalloproteinases or TIMPs) VEGF seems to play an important role in the development of osteoarthrosis. In late stages of osteoarthritis, invasion of blood vessels from the subchondral growth plate, synovitis with angiogenesis and osteophyte growth is observable. Several studies have revealed a central role for VEGF in all these phenomena. In order to investigate whether VEGF participates in early changes of OA or may even possess characteristics of a marker of OA, we developed an experimental posttraumatic OA New Zealand White rabbit animal model.

*Materials and methods:* In four skeletally mature New Zealand White rabbits, OA was induced by joint instability after transsection of the anterior cruciate ligament in both knees. After eight weeks the animals were killed. OA was verified histologically using the Mankin scale. Expression of VEGF was detected by immunohistochemistry and RT-PCR. Proteoglycans were evaluated by using HE and safranin-O staining. Four non-surgically treated animals acted as a control.

*Results:* The mean Mankin score was 5.11 (±2.14), corresponding to a moderate OA. VEGF and VEGF transcripts were detectable in the cartilage of early experimental posttraumatic OA rabbits. Control samples remained negative for VEGF mRNA and protein.

*Discussion:* The results of this study are promising concerning the role of VEGF as a diagnostic marker. VEGF could further be participated in early changes of OA. A therapeutic approach by modulation of VEGF production could be a possibility for the future.



## 1. Introduction

Osteoarthritis (OA) is an important disease due to the longer life expectancy of an increasingly large aging population. It is estimated that by the year 2020, nearly 60 million Americans will suffer from some kind of OA (Hinton et al., 2002; Lawrence et al., 1998). Degradation of cartilage leads to the development of fissures, fibrillation and the loss of joint surface. Subchondral bone sclerosis and formation of osteophytes are secondary changes accompanying the process. Even the synovial membrane is involved in later stages. The synovium is at least partially responsible for the inflammatory reaction leading to joint pain. Although articular cartilage is an avascular tissue, due to the production of angiogenesis inhibitors (Horner et al., 1999; Pufe et al., 2004a), it is a liable target for capillary invasion and angiogenesis with synthesis of angiogenic activators (Alini et al., 1996; Descalzi Cancedda et al., 1995). Vascular endothelial growth factor (VEGF) is a very potent angiogenic peptide and plays an important role in embryonic vasculogenesis and angiogenesis in normal growth (Aoyama et al., 2004; Gerber et al., 1999; Horner et al., 2001; Pufe et al., 2001a). Production of VEGF occurs in chondrocytes of OA-affected joints (Enomoto et al., 2003; Neufeld et al., 1999; Pufe et al., 2001b) as well in mechanical overload (Pufe et al., 2004b) – a known predisposing factor for the development of OA. VEGF was found in synovial tissue of patients with primary or secondary inflammatory joint diseases (Fava et al., 1994; Fay et al., 2006; Pufe et al., 2001b,c).

There is limited literature regarding the expression of VEGF on protein and mRNA levels in early OA stages. Therefore we

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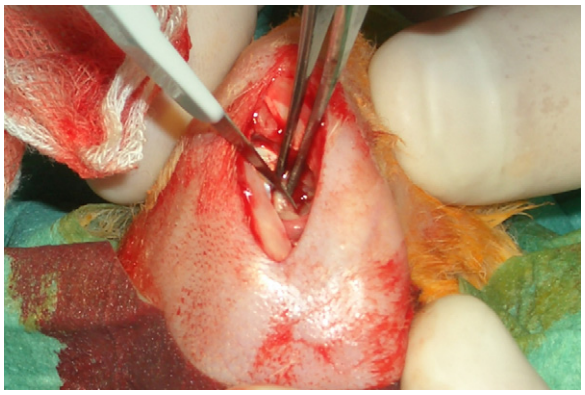

**Fig. 1.** Dissection of the anterior cruciate ligament.

investigated the expression of VEGF in a rabbit model with an early stage of OA determined according to the Mankin scale (Mankin et al., 1971).

## 2. Materials and methods

### 2.1. Animal model

The study was authorized by the locally responsible ethics committee and authorities (AZ G17/2004). The study was performed on eight knees of four skeletally mature 20 month old male New Zealand White rabbits (Peter Rollíe, Oelde, Germany) with a mean body weight of 3366 g (±188 g). All animals were kept in plastic boxes (100 cm × 70 cm × 40 cm) at a room temperature of 20–22 °C, starting two weeks preoperatively. The day/night period was 12 h. Animals had free access to water and food. For the operative procedure, anaesthesia was administered using a mixture of 0.5 ml (20 mg/ml) xylacine-hydrochlorate (Rompun Vet., Bayer, Germany) and 1.5 ml (100 mg/ml) ketamine-hydrochloride (Sanofi-CEVA GmbH) by intramuscular injection. About 5 min later, sufficient anaesthesia was achieved. Hind legs were shaved and disinfected with 80% alcohol for 5 min and a sterile operative field was prepared with the animals lying supine on the operating table. Breathing and pupillary reflexes were monitored. An antero-medial incision was made and the knee cup was laterally dislocated so that the anterior cruciate ligament (ACL) could be clearly seen. Transsection of the ACL was performed with a 2 mm resection to prevent spontaneous healing (Fig. 1). To verify knee instability the Lachman-test was carried out intraoperatively. The joint was rinsed with physiological 0.9% sodium-solution. The capsule and skin were sutured (Vicryl 5–0, Johnson & Johnson Medical Products GmbH, Germany). All animals received a single intramuscular dose of broad-spectrum antibiotics (Tardomyocel; Bayer, Leverkusen, Germany) as prophylaxis against infection. Postoperatively, all animals were allowed free movement in their boxes with daily check-ups for signs of wound healing problems or infection. With ongoing instability of the knee, OA developed over time. After eight weeks, the animals were killed. Cartilage from four male 20 month old normal rabbits without sham operation and ACL transsection was also harvested. Lung tissue from the central part and from the periphery served as positive control.

### 2.2. Histology

Tissue samples were fixed in 4% paraformaldehyde for 2 days. After decalcification in buffered EDTA (20% EDTA, pH 7.4), the samples were dehydrated and embedded in paraffin. Sections were cut at a thickness of 5 μm, mounted on poly-L-lysin-coated glass slides, deparaffinized in xylene, and washed 3 times with distilled water and then with Tris-buffered saline (TBS; pH 7.5) for 2 min each (washing procedure). Sections were stained with safranin-O or with hematoxylin and eosin (H&E) to evaluate histologic changes in the cartilage and bone tissue according to the Mankin scale based on structure of the cartilage, cells, safranin-O-staining and integrity of the chondral-bone border with a scoring range from 0 to 14 (best to worst) related to instability of the knee joint (Fig. 1).

### 2.3. Immunohistochemistry

For immunohistochemistry, sections were dewaxed, incubated with testicular hyaluronidase (2 mg/ml in PBS, pH 5.0, for 30 min at room temperature) and pronase (1 mg/ml in PBS, pH 7.4, 30 min at room temperature), then immunostained with anti-VEGF (1:40 in Tris-buffered saline, 60 min; sc-7269 mouse monoclonal $IgG_{2a}$, Santa Cruz Biotechnology, CA, USA).

### 2.4. RT-PCR

For RT-PCR, −80 °C frozen samples (10 mg) were crushed in an agate mortar under liquid nitrogen, homogenized in 5 ml "peqgold RNA Pure" solution (peqLab Biotechnologie, Erlangen, Germany) with a Polytron homogenizer, insoluble material was removed by centrifugation (12,000 × *g*, 5 min, 4 °C). RNA was isolated as described by the manufacturer (phenol–guanidinium thiocyanate method). We isolated up to 100 ng total RNA from 10 mg cartilage tissue. Crude RNA was purified by isopropanol and repeated ethanol precipitation, and contaminating DNA was destroyed by digestion with RNase-free DNase I (20 min 25 °C; Boehringer, Mannheim, Germany). After inactivation of the DNase (15 min 65 °C), cDNA was generated with 1 μl (20 pmol) oligo $(dT)_{15}$ primer (Amersham Pharmacia Biotech, Uppsala, Sweden) and 0.8 μl superscript RNase $H^-$ reverse transcriptase (Gibco, Paisley, UK) for 60 min at 37 °C. For PCR, 4 μl cDNA was incubated with 30.5 μl water, 4 μl 25 mM $MgCl_2$, 1 μl dNTP, 5 μl 10x PCR buffer, and 0.5 μl (2.5 U) Platinum *Taq* DNA polymerase (Gibco) and 2.5 μl (10 pmol) of each primer pair. The following primers/conditions were applied: VEGF, 5′-GTG GAC ATC TTC CAG GAG TA-3′ (sense) and 5′-GGT CTG CAT TCA CAT TTG TTG-3′ (antisense) with 35 cycles performed at 56 °C annealing temperature yielding a 221 bp product. The RT-PCR for glycerinaldehyde-3-phosphatedehydrogenase (GAPDH), yielding a product of 465 bp, was used to control equal amounts and intactness of the mRNA.

## 3. Results

In the osteoarthrosis group the anterior cruciate ligament was transsected in all but one knee. After eight weeks a moderate osteoarthrosis was detectable according to the Mankin scale.

The average Mankin score was 5.11 ± 2.14 (mean ± SD), corresponding to moderate OA as ascertained in the histological stains. In HE staining a clustering of chondrocytes was detectable (Fig. 2a). The safranin-O-staining revealed a loss of proteoglycans in superficial layers of articular cartilage (Fig. 2b). Pannus formation was not seen in any animal of either group. The Mankin score of the control group was 1.18 (±0.63 SD). The Mann–Withney-*U*-test revealed a significantly higher Mankin score in the OA group ($p = 0.01$) (Fig. 2c).

VEGF protein was detectable in animals with unstable femorotibial joints using immunohistochemistry (Fig. 3a). Interestingly, the chondrocytes of the clusters were strongly stained for VEGF (Fig. 3a arrows). In healthy animals, no VEGF protein was detectable (Fig. 3b). Negative controls were performed by absorption of the primary antibody with recombinant VEGF and revealed no staining (not shown).

VEGF transcripts were detectable in the cartilage of the treated animals. Compared to control cartilage tissue (Fig. 4, lane 4) the