Expression of vascular endothelial growth factor during healing of the meniscus in a rabbit model

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Our aim was to investigate vascular endothelial growth factor (VEGF) expression after lacerations of a meniscus in a rabbit model. Specimens of meniscus were examined using immunohistochemistry, enzyme-linked immunoassay and the reverse transcription polymerase chain reaction after one, two, five or ten weeks. In the periphery of the meniscus 90% of the lacerations had healed after five and ten weeks, but no healing was observed in the avascular area. Expression of VEGF protein and VEGF mRNA was found in the meniscus of both the operated and the contralateral sites but both were absent in control rabbits which had not undergone operation. The highest expression of VEGF was found in the avascular area after one week (p < 0.001). It then lessened at both the vascular and avascular areas, but still remained greater in comparison with the control meniscus (p < 0.05). Despite greater expression of VEGF, angiogenesis failed at the inner portion. These findings demonstrated the poor healing response in the avascular area which may not be caused by an intrinsic cellular insufficiency to stimulate angiogenesis.

The meniscus has a limited intrinsic capacity for healing because only 10% to 25% of its periphery has a blood supply.1,2 Several studies have shown that tears in the avascular area do not heal successfully by suture repair alone.3-5

Angiogenesis, the formation of new blood vessels from pre-existing capillaries, is essential for healing of injured tissue. It is controlled by a variety of mitogenic and chemotactic peptides which act on invading endothelial and smooth muscle cells. The most potent angiogenic factor known is vascular endothelial growth factor (VEGF), sometimes called the vascular permeability factor (VPF), which was originally identified as a heparin-binding angiogenic peptide secreted by tumour cells.6,7 VEGF is a selective endothelial cell mitogen which promotes angiogenesis in vivo and renders the microvasculature hyperpermeable to circulating macromolecules.8 It is also chemotactic for monocytes and is a procoagulant.8 The two signalling tyrosine kinase receptors, the Fms-like tyrosine kinase receptor (FLT-1, VEGFR-1) and the kinase insert domain-containing receptor KDR (VEGFR-2/FLK-1), selectively bind VEGF.9-11

In normal tissues, VEGF is expressed during embryogenesis,12-14 in the epiphyseal plate during skeletal growth,15 and at a few sites in the adult human where the formation of new blood vessels occurs such as in the corpus luteum or endometrium.8 Expression of certain genes during development can also occur later on in the diseased state. In adults, apart from its association with tumours, VEGF has also been detected in the synovial tissue of patients with rheumatoid arthritis,16 in osteoarthritic cartilage17 and in degenerative tendon tissue.18 We are not aware of any published studies which have investigated the expression of VEGF during healing of the meniscus.

Our study focuses on VEGF in meniscal tissue and investigates its expression during healing of the meniscus in a rabbit model. We hypothesised that higher levels of expression of VEGF at the periphery of the meniscus may contribute to a better healing response and that little or no expression may be expected in the avascular area; its absence may contribute to the poor healing response.

Materials and Methods

The study had the approval of the local government animal rights protection authorities in accordance with the National Institute of Health guidelines for the use of laboratory animals. We used 45 skeletally mature New Zealand White rabbits with a mean age of 6.3 months (5 to 7) and a mean height of 3.6 kg (2.9 to 4.6). They were anaesthetised by intramuscular injections of ketamine hydrochloride (60 mg/kg; Ketanest, Parke Davis GmbH, Ber-
lin, Germany) and 2% xylazine hydrochloride (4 mg/kg; Rompun, Bayer Vital GmbH & Co KG, Leverkusen, Germany). The rabbits were placed supine and the knee approached through a medial parapatellar incision. The patella and the fat pad were displaced laterally to display the medial meniscus. The anterior horn of the medial meniscus was subluxed anteriorly and a longitudinal laceration, 5 mm in length, was made in the anterior horn where the meniscus has a mean radial dimension of 4 to 5 mm. The laceration was made to resemble closely a bucket-handle tear, either 1 mm parallel to the meniscus on the rim in the vascular portion (group A) or 1 mm parallel to the inner circumference in the avascular portion (group B). The wound was closed in layers using 4-0 Vicryl for the capsule and 3-0 Prolene for the skin. The animals were mobilised in a cage after operation without immobilising the hind limbs.

A total of 44 New Zealand White rabbits were killed by the administration of sodium pentobarbital (200 mg/kg/ body-weight; Narcopen, Pantobarbital-Na, Rhone Merieux GmbH, Laupheim, Germany). Five rabbits in each of the two groups were killed at intervals of one, two, five and ten weeks, except in group B after ten weeks when one rabbit had died during the early post-operative stage. The remaining five rabbits served as a control group and did not undergo operation. The medial menisci of both the operated and the contralateral knee were harvested. Samples were taken from the area of the lesion and from the same area of the contralateral meniscus in each rabbit.

For conventional light microscopy, sections were cut at 4 µm, mounted on gelatin-coated slides, and stained with haematoxylin and eosin and Goldner’s stain. Immunohistochemical staining, enzyme-linked assay (ELISA), and the reverse transcriptase-polymerase chain reaction (RT-PCR) were also carried out. **Immunohistochemistry.** For immunohistochemical staining the meniscal samples were fixed in 5% formalin, embedded in paraffin, sectioned and mounted on Histo-Bond Adhesion Macro Slides (Paul Marienfeld GmbH & Co KG, Lauda-Koeningshofen, Germany). The slides were dewaxed in a descending concentration of alcohol and each slide was irradiated twice for five minutes at 750 W in a microwave oven with 3% hydrogen peroxide in 0.01 M sodium citrate solution Macro Slides (Paul Marienfeld GmbH & Co KG, Laupheim, Germany). Five rabbits in each of the two groups were killed at intervals of one, two, five and ten weeks, except in group B after ten weeks when one rabbit had died during the early post-operative stage. The remaining five rabbits served as a control group and did not undergo operation. The medial menisci of both the operated and the contralateral knee were harvested. Samples were taken from the area of the lesion and from the same area of the contralateral meniscus in each rabbit.

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For visualisation of blood vessels a monoclonal antibody against factor VIII (1:80 in TBS, Santa Cruz) was used. Factor-VIII immunoreactivity was analysed semiquantitatively by a pathologist, who was not involved in the study, according to the method stated by McDougall et al. At a magnification x200, the extent of vascularity or the number of vessels around the lesion within the microscopic field was estimated to be either absent (0), few (+), many (++), or abundant (+++). Five sections were examined from the injured and the contralateral menisci of each of the five animals in each group and at each time interval (one, two, five and ten weeks) except for group B which after ten weeks had only four animals.

**Enzyme-linked immuno-assay (ELISA).** For the ELISA technique, frozen tissue samples were crushed in an achate mortar under liquid nitrogen, and homogenised in 150 mM NaCl, 20 mM Tris/HCl-buffer at pH 7.4. A soluble fraction was obtained by centrifugation (48 000 x g, 60 minutes), and aliquots (100 µl) were analysed by a sandwich ELISA (R&D Systems, Minneapolis, Minnesota) which detects all VEGF splice forms. Human recombinant VEGF (PreproTech, Rocky Hill, New Jersey) served as the standard. **Reverse transcription-polymerase chain reaction (RT-PCR).** The sediment of the homogenised tissue was used for RT-PCR. Isolation of RNA was achieved using the phenol-guanidinium-thiocyanate method. The samples were purified in isopropanol and alcohol. After inactivation of the DNA using RNase-free DNase (20 minutes at 25°C, Roche, Mannheim, Germany) cDNA was generated using 1 µl (20 pmol) of oligo (dT)15 primer (Amersham Pharmacia Biotech, Uppsala, Sweden) and 0.8 µl of superscript RNAse H cReverse transcriptase (Gibco, Paisley, UK) for 60 minutes at 37°C. For the PCR, 4 µl of cDNA were incubated with the following primers (2.5 µl each containing 10 pmol), non-selective for all VEGF splice variants 5'-CTA-CCG-GGC-CAC-TAC-TGC-C-3' (sense) and 5'-CCC-TGG-TGA-GGT-ATT-ATG-CG-3' (antisense) yielding a 203bp fragment (35 cycles, annealing temperature 60°C). The RT-PCR for glycerinaldehyde-3-phosphatedehydrogenase (GAPDH) yielding a product of 465 bp was used to control equal amounts and the intactness of the mRNA. **Statistical analysis.** The results are reported as mean values and the SEM. One-way analysis of variance was used to compare expression of VEGF between the lacerations at the
periphery and those of the central portion of the meniscus after one, two, five and ten weeks. The post-hoc Scheffé test was used when the analysis of variance showed significance. The level of statistical significance was set to an alpha level of p < 0.05.

Results
All the arthrotomy incisions had healed after five days without any sign of infection and the rabbits showed normal activity in their cage after operation.

Gross examination. After one and two weeks, no healing was observed at the periphery or in the central area of the meniscus. Synovial hypertrophy was noticed in specimens with a lesion at the periphery. In five specimens hypertrophic synovial tissue covered the meniscal lesion. Five weeks after injury, healing was observed in four of the five specimens at the periphery of the meniscus. Synovial hypertrophy and the appearance of small vessels were seen on the surface of the meniscus. After ten weeks all the lacerations at the periphery had healed with 90% doing so between five and ten weeks. No healing was observed in those in the central area of the meniscus.

Histological assessment of healing of the meniscus, vascularity and VEGF immunostaining. In the first two weeks of healing of the meniscal lesions at the periphery showed an increased vascularity as evidence by factor-VIII immunostaining.

In group A after one week, four specimens showed abundant and one had many vessels. After two weeks, three specimens had abundant and two had many vessels. After five weeks, three specimens showed many and two showed few vessels. Finally, after ten weeks two specimens showed many vessels and three had few. No vascularity was seen in specimens with lacerations in the avascular inner area (group B) by up to ten weeks after operation.

Increased cell density was found on histological specimens when compared with normal rabbit meniscal tissue. Specimens with their laceration at the periphery contained an inflammatory cell infiltrate consisting of fibroblasts, capillary endothelial cells and other inflammatory cells. All these cells labelled strongly positive for VEGF (Fig. 1a). Immunostaining in the control rabbits did not produce a positive response (Fig. 1b). The negative immunocontrol remained negative in the meniscal lesion (Fig. 1c). The endothelial cells of the blood vessels expressed the proliferation marker Ki-67. After two weeks, the meniscus was still not healed. The adjacent synovial membrane was thickened and synovial lining cells stained strongly for VEGF. Subsynovial vascular proliferation also showed marked immunostaining for VEGF.

In specimens from the inner portion, fibrochondrocytes were strongly immunostained with the VEGF antibody. VEGF immunostaining persisted until the tenth week without signs of healing. The synovial lining cells of specimens with their laceration in the avascular portion were also strongly immunopositive for VEGF. In specimens from the contralateral side VEGF immunostaining was positive in fibrochondrocytes, fibroblasts, and endothelial cells of the periphery of the meniscus. By contrast, rabbits which had not undergone operation did not show expression of VEGF either at the periphery of the meniscus or at the inner portion.

Immunostaining for the VEGF receptors VEGFR-1 (Flt-1) and the VEGFR-2 (Flk-1/KDR). Immunostaining for the VEGF receptors VEGFR-1 and VEGFR-2 was positive in all animals undergoing operation but not in those which did not (Figs 2 to 4). Immunostaining for VEGFR-1 was restricted to the vascular endothelial cells of capillaries at the periph-
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ERY of the meniscus (Fig. 2a). In larger vessels such as arterioles or venules smooth muscle cells in the vessel wall were also VEGFR-1-positive. The staining pattern of VEGFR-2 was positive for vascular endothelial cells, inflammatory cells, single fibrochondrocytes and some fibroblastic cells of the peripheral reparative tissue (Fig. 3a). In addition, some of the fibrochondrocytes of the inner portion stained positively for VEGFR-2 (Fig. 4a). The control immunostaining was negative for both VEGFR-1 (Fig. 2b) and VEGFR-2 (Figs 3b and 4b). Negative controls were also carried out for both receptors as shown from specimens of the meniscal lesion (Figs 3c and 4c).

**VEGF concentration during meniscal healing measured by ELISA.** Significantly higher expression of VEGF was found in specimens with meniscal lesions in comparison with the contralateral side (p < 0.05; Fig. 5). By contrast, no VEGF was found in specimens from animals which had not undergone the operation.

After seven days the mean concentration of VEGF at a lesion of the central portion was 1537 ± 187.4 pg/ml, twice that of lesions at the periphery (727.2 ± 25.8 pg/ml) (p < 0.001). In specimens with lacerations in the central avascular portion, the concentration of VEGF decreased gradually after the first, second, fifth and tenth weeks (p < 0.05). There was a significant decline in the expression of VEGF between the first and second weeks and the fifth and tenth weeks in specimens from animals which had not undergone the operation.

**Reverse transcription-polymerase chain reaction.** Samples from animals undergoing operation showed a PCR product with 203 bp corresponding to VEGF at the periphery (3) and inner portion (2) of the menisci with lacerations. The control of intactness of the mRNA shows the GAPDH band at 465 bp (Fig. 6). The meniscal specimens from the contralateral side (1) had only a weak band of 203 bp corresponding to VEGF. In adult meniscal tissue of animals which had not been operated on no PCR signal for VEGF was detectable.

**Discussion**

Longitudinal lacerations were made at either the periphery of the meniscus, the vascular portion, or close to the central rim, the avascular part. After five weeks four of the five
lacerations and after ten weeks all had healed at the periphery of the meniscus. No healing was found in the inner portion. Huang et al.\textsuperscript{20} also investigated the healing response of experimentally created lacerations of the meniscus in rabbits and found significantly better healing at the periphery. Similar results have also been reported by other authors.\textsuperscript{21,22}

Increased expression of VEGF was observed in specimens with lacerations at the peripheral and central portion compared with the intact meniscus on the contralateral side. Peak levels of VEGF were found after seven days in both areas, followed by a gradual decline in expression. In contrast to our initial hypothesis, the level of VEGF was more than twice as high after one week in specimens with lacerations at the central portion than in those with lacerations at the periphery. Previous studies have shown that hypoxia and growth factors synergistically up-regulate expression of VEGF in various types of cell and this may explain the higher level of VEGF in the avascular portion at the early phase of healing.\textsuperscript{23-25}

Expression of VEGF did not achieve levels which were sufficient to cause formation of new vessels in the central avascular portion of the meniscus. However, a study by Phillips et al.\textsuperscript{26} has shown that the formation of new capillaries in avascular tissue may be induced by high levels of VEGF. Human VEGF\textsubscript{165} was diluted in Dulbecco’s phosphate, dried and implanted in the rabbit cornea. After five to seven days, capillaries had formed between the limbus and the site where the dried VEGF had been implanted. The effective concentration of VEGF (200 to 1000 ng) was much higher than that found in our study. Whether dose-dependent levels of VEGF may also influence healing of the meniscus at the inner area needs to be investigated further.

However, a lower concentration of VEGF in the peripheral vascular portion after one week causes proliferation of endothelial cells, suggesting the presence of a strong antiangiogenic stimulus in the inner avascular portion. Both stimulatory and inhibitory peptides seem to control angiogenesis. The expression of antiangiogenesis factors may be involved in preventing vascular ingrowth in order to maintain avascularity in the inner portion of the meniscus. Vascular meniscal tissue and its biomechanical properties provide the environment for normal meniscal function. Moses, Sudhalter and Langer\textsuperscript{27} and Moses et al.\textsuperscript{28} showed that cartilage contains endogenous inhibitors of angiogenesis. Troponin 1, a subunit of the troponin complex, has been identified as a potent and specific inhibitor of angiogenesis in hyaline cartilage. Similar mechanisms could prevent the formation of capillaries in fibrocartilage. Angiostatin and endostatin are also anti-angiogenic factors which have antiproliferative effects, reducing the number of endothelial cells and the amount of formation of new vessels.\textsuperscript{29} A lack of the intrinsic capacity of the inner portion of the meniscus may be another reason for missing angiogenesis. This may be because the cells are unable to extract themselves from the surrounding matrix. Lack of scaffolding could inhibit cells at the inner area from creating new blood vessels.

There are no pre-existing vessels in the central portion of the meniscus. During angiogenesis endothelial cells normally spread out into the surrounding tissue from pre-existing vessels. Growth factors or cytokines may reach a lesion at the periphery via diffusion from the inflamed synovium. Ochi et al.\textsuperscript{21} showed that meniscal rasping after a sharp longitudinal laceration promoted healing of a lesion at the inner portion and a significantly higher expression of PDGF, IL-1 and TGF-\( \beta \). Other studies have shown that extrinsic factors such as fibrin with\textsuperscript{10} or without cells,\textsuperscript{31} synovial tissue\textsuperscript{22,32,33} or type-I collagen scaffolds\textsuperscript{34} also promote healing of meniscal lesions in the avascular portion.

Expression of VEGF was also noticed in fibroblastic cells and fibrochondrocytes of menisci with lacerations at the periphery. Not all of the fibrochondrocytes were positively immunostained in the inner portion of the meniscus. Ghalbly, Lalonde and Wedge\textsuperscript{35} identified differences between the chondrocytes in the superficial and middle meniscal zone. Beside the differences in the chondrocytes they also found fibroblasts and myofibroblasts which are only encountered in injured menisci near the damaged site. These cells may not express VEGF.

Expression of VEGF was also noticed in the control samples from the contralateral side which had not had an operation. A stress-induced pattern secondary to differences in mobility when the rabbits were protecting their injured knee and thus overstressing their contralateral normal knee may have induced expression. Zheng et al.\textsuperscript{16} have shown that expression of VEGF in cells is sensitive to mechanical stimuli. The application of intermittent cyclic stretching increased expression of VEGF in cardiac fibroblasts.

The proliferative endothelial response to VEGF is mediated by the signalling tyrosine kinase receptor VEGFR-2 which selectively binds VEGF.\textsuperscript{12,37} In our study VEGFR-2 immunoreactivity was not restricted to vascular endothelial...
and smooth muscle cells. Surprisingly a small number of fibrochondrocytes at the peripheral and inner portion was labelled strongly positive for VEGF-2 suggesting other para- or autocrine effects of VEGF in the meniscal tissue or the presence of different types of cell at the site of the lesion, as described by Ghadially et al. Expression of VEGF-2 is also exhibited by other cartilage cells such as hypertrophic chondrocytes from the growth plate, and chondroclasts, and osteoarthritic chondrocytes. In our meniscal healing model immunostaining for VEGFR-1 was restricted to vascular endothelial cells and smooth muscle cells of the capillaries at the periphery of the meniscus of the operated animals. The VEGF-1 receptor is chemotactic for monocytes, and when stimulated on endothelial cells modifies the response to VEGF-2.

The effect of trauma to the knee during the surgical procedure in our animal model remains unclear in terms of meniscal healing. The lesion was created artificially and an arthrotomy, which itself is traumatic to the joint, was required. Our study has shown that VEGF protein and mRNA are strongly expressed by endothelial cells and fibrochondrocytes of the meniscal tissue, in both the vascular and avascular portions. Despite a high concentration of VEGF, lesions in the avascular portion failed to heal.

Wound healing involves complex mechanisms and further studies are required to investigate the effect of other angiogenic or antiangiogenic factors during meniscal healing and their effect on cell activity as well as their interaction with the extracellular matrix.

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No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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