Original Article

Vascular Endothelial Growth Factor (VEGF) is Expressed During Articular Cartilage Growth and Re-expressed in Osteoarthritis

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Abstract

Introduction: Vascular endothelial growth factor (VEGF) is expressed in osteoarthritic articular cartilage. However, the pattern of VEGF expression throughout the whole life cycle of articular cartilage is not well elucidated. The aim of the study was to investigate the spatiotemporal expression of VEGF and its receptors, vascular endothelial growth factor receptor-1 (VEGFR1) and vascular endothelial growth factor receptor-2 (VEGFR2), in articular cartilage during growth, maturation and degeneration, using the guinea pig model of spontaneous osteoarthritis. Materials and Methods: Sections of tibial plateaus aged 2, 6 and 12 months were obtained, representing growing, mature and osteoarthritic cartilage respectively. Expression of VEGF and its receptors was determined by immunohistochemistry and in situ hybridisation. Results: At 2 months, VEGF and its receptors were expressed in chondrocytes within the superficial layer of the articular cartilage. At 6 months, no expression of VEGF and its receptors was noted. In the 12-month-old specimens, VEGF and its receptors were expressed in chondrocytes within articular cartilage that exhibited osteoarthritic changes (medial tibial plateaus), but not in the histologically normal lateral plateaus. Conclusion: This spatiotemporal distribution of VEGF and its receptors suggests that VEGF is expressed during articular cartilage growth, becomes quiescent at maturity, and is re-expressed in osteoarthritis.

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Introduction

Growth factors are polypeptides that direct cells to proliferate, differentiate, migrate or produce matrix.[1] They exert their effects by interacting with specific receptors on the surfaces of cells. A number of these growth factors have been shown to play important roles in articular cartilage physiology by regulating the behaviour of chondrocytes.[2] These include insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), and bone morphogenetic proteins (BMPs). For instance, IGF is an important stimulatory factor for cartilage matrix synthesis, while bFGF is known to strongly stimulate chondrocyte proliferation.[3] The inhibition of IGF and TGF-β has been implicated in the pathogenesis of osteoarthritis.[4] The improved understanding of growth factor biology in articular cartilage has led to investigations looking at their potential for enhancing cartilage repair. For example, BMP-2 has been shown to improve the repair of full-thickness osteochondral defects in rabbits.[5]

More recently, vascular endothelial growth factor (VEGF) has been reported to be present in articular cartilage.[6-8] VEGF is more widely known for the key part it plays in angiogenesis, where it stimulates the proliferation and migration of endothelial cells.[9,10] Angiogenesis occurs in a wide variety of physiological processes, such as tissue remodelling, and pathological conditions, such as malignancy. In physeal cartilage, VEGF is known to play an essential role in endochondral bone formation by coupling angiogenesis with hypertrophic cartilage remodelling and ossification.[11]

Pufe at al[6] were the first to report the presence of VEGF in articular cartilage. They looked at cartilage samples from normal and osteoarthritic adult human knee joints, and found VEGF to be expressed in osteoarthritic cartilage, but not in normal cartilage. Pfander at al[7] and Enomoto et al[8] reported similar expressions of VEGF in osteoarthritic articular cartilage. Various authors have gone on to suggest that VEGF is involved in the pathogenesis and

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progression of osteoarthritis.[12,13]

Little, however, is known about the expression of VEGF in articular cartilage during other phases of development, such as growth and maturation. For instance, its expression in growing articular cartilage has not been well elucidated, and there is conflicting evidence as to whether VEGF is present in normal mature articular cartilage. While Pufe at al[6] found VEGF not to be expressed in normal articular cartilage, Pfander et al[7] found it to be present in all layers of normal adult cartilage. The sequential distribution of VEGF during the growing, mature and degenerate phases of articular cartilage development is also unclear.

This study was carried out to ascertain the spatiotemporal expression of VEGF and its receptors, vascular endothelial growth factor receptor-1 (VEGFR1) and vascular endothelial growth factor receptor-2 (VEGFR2), in articular cartilage during growth, maturation and degeneration.

Materials and Methods

Animals and Tissues

Male Dunkin Hartley guinea pigs were used as the model for this study. These animals develop spontaneous age-related osteoarthritic changes in their knee joints, particularly in the medial tibial plateau. Moderate-to-severe changes are seen when the guinea pigs are 12 months of age.[14]

Guinea pigs of the following ages were used – 2 months, 6 months and 12 months. These age-points were chosen to study growing, mature and osteoarthritic articular cartilage, respectively. There were 4 guinea pigs in each group. Ethical guidelines pertaining to the use of animals for experimental study were followed, and approval from our hospital's Institutional Review Board was obtained.

Tibial plateaus were harvested from the knee joints of the guinea pigs, following sacrifice by carbon dioxide inhalation. The specimens were fixed in 10% formaldehyde and decalcified in 30% formic acid. They were then dehydrated, embedded in paraffin and sectioned coronally into 7-micrometer thick slices.

Immunohistochemistry

The sections were deparaffinised, rehydrated and treated with hydrogen peroxide to block endogenous peroxidase. They were also treated with Ultra V Block (Lab Vision; Fremont, CA, USA) to reduce non-specific binding. They were then treated with rabbit polyclonal antibodies against VEGF and VEGFR2 (Neomarkers; Fremont, CA, USA), as well as mouse monoclonal antibodies against VEGFR1 (Chemicon; Temecula, CA, USA). The working concentration of each antibody was 5ug/mL, and the sections were incubated for 30 minutes at room temperature.

After this, they were incubated with biotinylated secondary antibodies and subsequently peroxidase-conjugated streptavidin (Lab Vision; Fremont, CA, USA). Colour was developed with diaminobenzidine. Counterstaining was performed with haematoxylin. Controls were generated by omitting the primary antibody or replacing the primary antibody with non-immune antibodies (Dako; Glostrup, Denmark). Sections were examined under light microscopy for localisation of VEGF and its receptors within the articular cartilage.

In Situ Hybridisation

The sections were deparaffinised and rehydrated. They were then pre-treated with Proteinase K and Peroxidase blocking solution. In situ hybridisation was performed using an oligonucleotide probe labelled with fluorescein isothiocyanate (FITC) (GeneDetect; Auckland, New Zealand). The anti-sense sequence of the probe was 5'-TGA TCC GCA TGA TCT GCA TGG TGA TGT TGA ACT CCT CGG TGG GCA CAC - 3'. Incubation with the probe was performed at 42°C for 2 hours.

Hybridised sections were then incubated with anti-FITC antibodies conjugated to alkaline phosphatase and fluorescyl tyramide (Dako; Glostrup, Denmark). Colour was developed using NBT/BCIP. Counterstaining was performed with eosin. Controls were generated using a FITC-labelled sense probe (5'-ACT AGG CGT ACT AGA CGT ACC ACT ACA ACT TGA GGA GCC ACC CGT GTG-3'). Sections were examined under light microscopy for localisation of VEGF mRNA within the articular cartilage.

Results

Sections from the 2-month-old guinea pigs demonstrated uniformly increased cellularity throughout the articular cartilage. This feature is typical of growing articular cartilage. VEGF immunostaining was consistently positive within the articular cartilage of the medial and lateral tibial plateaus in all animals. This staining was uniformly confined to the superficial layer of the cartilage, and no staining was noted in the middle and deep layers of the cartilage (Fig. 1A). A similar pattern of localisation was seen for VEGFR1 and VEGFR2 (Figs. 1B, 1C). In situ hybridisation also demonstrated VEGF mRNA expression within the superficial layer of the articular cartilage (Fig. 1D).

Sections from the 6-month-old guinea pigs showed the characteristic layered structure of normal mature articular cartilage. However, no immunostaining of VEGF or its receptors was noted within the articular cartilage in all animals (Figs. 2 A-C). In situ hybridisation similarly showed no expression of VEGF mRNA within the articular cartilage (Fig. 2 D).

In the sections of articular cartilage from the 12-month-old guinea pigs, degenerative changes were observed in the

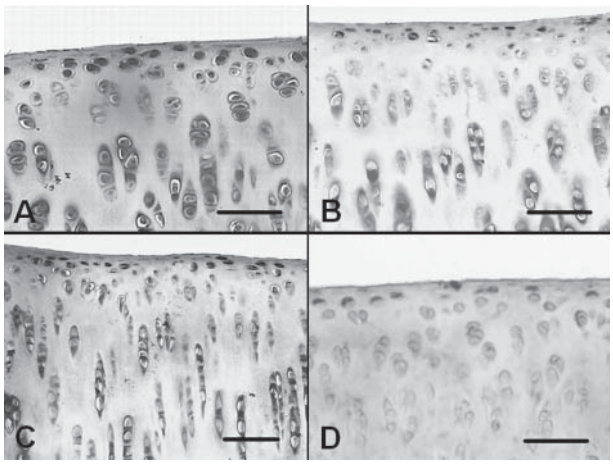

Fig. 1. VEGF-immunopositive cells (stained brown) are seen within the superficial layer of growing articular cartilage from a 2-month-old guinea pig (A). A similar pattern of localisation is seen for VEGFR1 (B) and VEGFR2 (C). VEGF mRNA-positive cells (stained brown) are seen in the superficial layer of growing cartilage from a 2-month-old guinea pig (D). Scale bar = 100 μm.

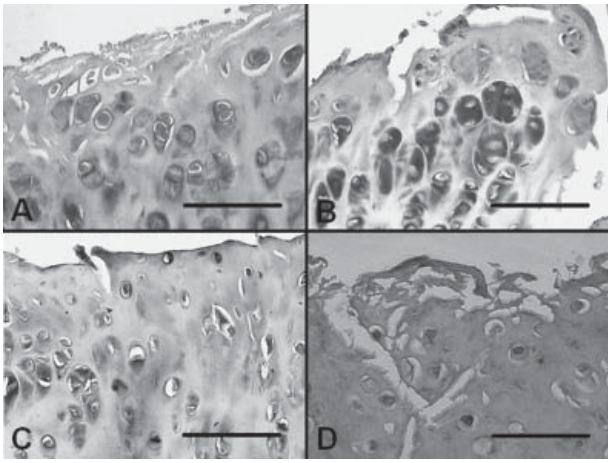

Fig. 3. VEGF-immunopositive chondrocytes are observed within the osteoarthritic cartilage of the medial tibial plateau from a 12-month-old guinea pig (A). A similar pattern of localisation is seen for VEGFR1 (B) and VEGFR2 (C). VEGF mRNA-positive cells are also visualised here (D). Scale bar = 100 μm.

medial tibial plateau. These changes included surface irregularities, fissuring and fibrillation. In contrast, no such changes were noted in the lateral plateau. VEGF-immunopositive chondrocytes were seen in the areas of cartilage breakdown in the medial tibial plateau. These chondrocytes were noted to be in the superficial and middle layers of the cartilage (Fig. 3 A). A similar pattern of localisation was seen for VEGFR1, VEGFR2 and VEGF mRNA (Figs. 3 B-D). In the lateral plateau, where the articular cartilage was unaffected by degenerative change, no such staining was observed (Figs. 4 A-D).

No VEGF immunostaining was noted when the primary antibody was omitted and when the primary

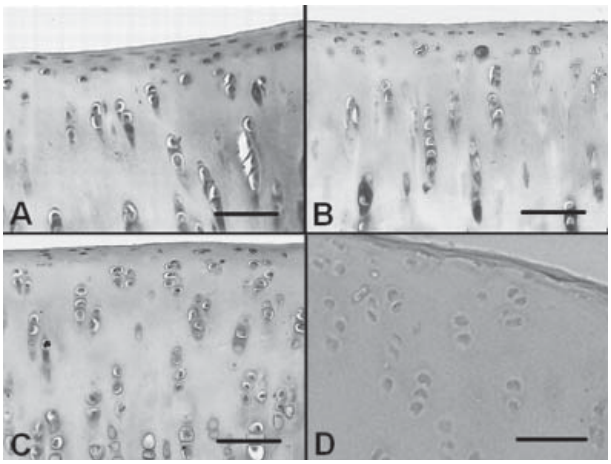

Fig. 2. VEGF (A), VEGFR1 (B), VEGFR2 (C) and VEGF mRNA (D) are not observed in normal mature articular cartilage from a 6-month-old guinea pig. Scale bar = 100 μm.

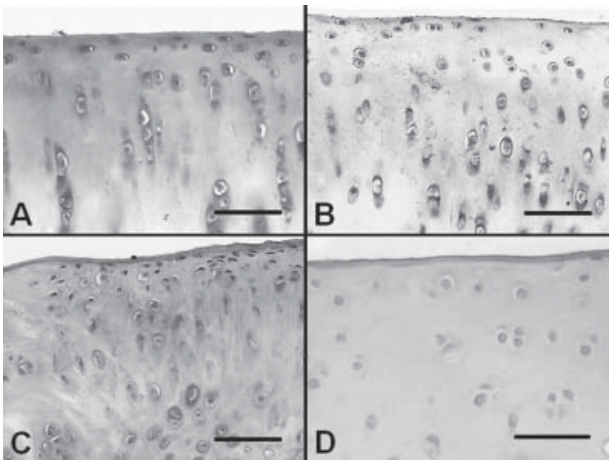

Fig. 4. VEGF (A), VEGFR1 (B), VEGFR2 (C) and VEGF-mRNA (D) are not observed in the lateral tibial plateau, which is unaffected by osteoarthritis. Scale bar = 100 μm.

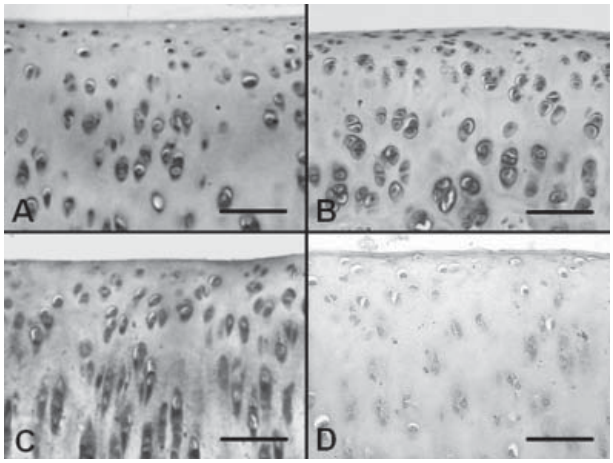

Fig. 5. No immunostaining was observed in the control sections for VEGF (A), VEGFR1 (B), and VEGFR2 (C) immunohistochemistry from a 2-month-old guinea pig. Similarly, no staining was observed when the sense probe was used during VEGF in situ hybridisation (D). Scale bar = 100 μm.

antibody was replaced by non-immune antibodies. In addition, no positive staining was noted when the sense probe was used during in situ hybridisation. (Figs. 5 A-D).

Discussion

Vascular endothelial growth factor (VEGF) is a 46 to 48 kD glycoprotein that exists in various isoforms. These isoforms are produced from a single VEGF gene by alternative splicing, and are made up of disulphide-linked homodimers.[9] VEGF exerts its effects by binding to the signalling tyrosine kinase receptors VEGFR1 and VEGFR2. These receptors are found primarily in endothelial cells, although they have also been identified in other cell types, such as trophoblasts, monocytes, haematopoietic stem cells and megakaryocytes.[8] Activation of the VEGFR2 receptor results in cell proliferation, whereas activation of the VEGFR1 receptor results in cell migration.[10] VEGF has been extensively studied with respect to its role in angiogenesis. Here it acts on endothelial cells to stimulate their proliferation and migration.[10] However, the specific biological activities mediated by VEGFR1 and VEGFR2 in articular cartilage chondrocytes have not been elucidated.

VEGF has previously been found to be expressed in osteoarthritic articular cartilage.[6-8] Our findings indicate that VEGF and its receptors are expressed in not just osteoarthritic articular cartilage, but in growing articular cartilage as well. They are not found in mature articular cartilage. In growing cartilage, we found both VEGF and its receptors to be expressed in chondrocytes within the superficial layer of the articular cartilage. The demonstration of VEGF and its receptors in the growing phase of articular cartilage suggests their potential involvement in articular cartilage growth and development. The presence of VEGF in the superficial layer, separated from deeper vascularised areas by a distinct VEGF-negative chondral layer, suggests a role in articular cartilage growth that is unrelated to angiogenic activity. The superficial layer has been shown to be the site of intense proliferative activity. There is also evidence to suggest that appositional growth occurs in this layer.[15,16] The localisation of VEGF within this layer suggests that it may be involved in the regulation of these processes. Further understanding of this phenomenon and its role in the development of normal articular cartilage may have implications for downstream applications such as tissue engineering of osteoarticular constructs, in which the exact replication of articular cartilage microstructure still remains an elusive goal.

In the articular cartilage from the 12-month-old guinea pigs, VEGF, as well as its receptors, were specifically found in the osteoarthritic medial tibial plateau, but not in the histologically normal lateral plateau. While previous studies have looked at osteoarthritic cartilage in isolation, we have looked at the expression of VEGF in osteoarthritic cartilage in relation to that in normal cartilage in the same specimen. In effect, the normal lateral plateau serves as an internal control to the osteoarthritic medial plateau. The differential distribution of VEGF and its receptors indicates that their expression is related to the pathological state of osteoarthritis, and is not simply an age-related phenomenon. There have been reports suggesting that VEGF may be involved in the formation of osteophytes and the induction of matrix-degrading catabolic factors in osteoarthritis.[17]

The spatiotemporal expression of VEGF and its receptors suggests that VEGF is expressed during articular cartilage growth, becomes quiescent at maturity, and is re-expressed in osteoarthritis. The expression of VEGF in articular cartilage during growth and degeneration raises the possibility of its involvement in cellular processes that are common to both these phases. Pufe at al[12] have shown that VEGF induces cell proliferation, stimulates matrix metalloproteinase (MMP) production, and inhibits tissue inhibitor of metalloproteinases (TIMP) production in immortalised human chondrocytes. Chondrocyte proliferation and matrix remodelling are processes that occur prominently in both growth and degeneration. MMP's are essential for matrix remodelling, and their expression in articular cartilage is known to be increased during periods of growth and degeneration.[12] Taken together, these findings suggest that VEGF may mediate the processes of chondrocyte proliferation and matrix remodelling during articular cartilage growth and degeneration, through the activation of its receptors VEGFR1 and VEGFR2.

It is intriguing to note that the above pattern of expression has been demonstrated in other pathophysiological processes as well, such as cancer, where genes that are expressed during early development, become quiescent at maturity and are subsequently re-expressed in the disease state. The findings of this study pave the way for further work to elucidate the molecular mechanisms by which VEGF may modulate articular cartilage growth and degeneration.

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