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Injection of vascular endothelial growth factor into knee joints induces osteoarthritis in mice

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SUMMARY

Osteoarthritis (OA) is a common joint disorder affecting *circa* 2% of the population.
Objectives: It has been suggested that secretion of vascular endothelial growth factor (VEGF) could play a role in the chain of events leading to OA.
Methods: In the present study, healthy mice were injected intra-articularly with VEGF.
Results: Shortly after the administration of VEGF, synovial hyperplasia, increased calcification of the articular cartilage and bone sclerosis were observed. Consequently, cartilage degradation characteristic of OA was found. These changes were seen to a lesser degree in the opposite knees of VEGF-injected mice and did not occur in the control mice.
Conclusions: The findings suggest an active role of VEGF in the pathogenesis of OA and render support to a possible role for subchondral bone sclerosis in the pathogenesis of cartilage degradation.

Introduction

Osteoarthritis (OA) is a prevalent, slowly progressing joint disorder clinically manifested by pain and disability. OA is characterized by synovitis, cartilage degeneration, subchondral bone sclerosis, and osteophyte formation. Articular cartilage degradation is a hallmark of OA. It should be pointed out that changes in the subchondral bone play a major role in the onset and progression of the disease[1–3]. Initiation of OA has been correlated with either excessive or alternatively, insufficient blood flow to the subchondral bone[4]. The latter may be associated with compromised delivery of nutrients and gas exchange with the articular cartilage[5].

In OA, osteoclastic resorption of the subchondral bone is followed by reduction of structural support to the articular cartilage[6,7]. Sclerosis associated contour changes result with a decrease in the mechanical, shock-absorbing support produced by the cartilage[1]. The damage to the articular cartilage is accompanied by de-novo formation of blood capillaries, initiation of osteophyte formation and calcification of the articular cartilage. Blood vessel formation may be accompanied by pro-apoptotic factors that lead to chondrocytic death[8]. In the synovial membrane, new vasculature could be responsible for edema and promotion of an inflammatory process[9] which among its other effects compromise joint lubrication[10]. Vascular endothelial growth factor (VEGF) is expressed in synoviocytes[10], macrophages[11] and in chondrocytes in human osteoarthritic joints[12]. VEGF has been shown to affect chondrocytic proliferation, apoptosis, and metabolism, leading to release of metalloproteinases (MMPs), as well as other catabolic mediators that degrade the cartilage matrix[13–15]. VEGF is expressed in the superficial zone of the cartilage disc, and has been shown to be secreted from mechanically overloaded chondrocytes[13] and in OA joints *in vivo*[16]. Another study showed that synovial fluid sourced from OA patients had 20-fold higher concentrations of VEGF in comparison to its level in healthy joints, thereby suggesting that VEGF takes part in OA development[17].

The present is a study of the effect of intra-articular injection, of exogenous VEGF, on mice knee joints. The histo-morphological structure of the joints was evaluated and graded at the cellular and structural levels. OA grading of the cartilage, subchondral calcified tissues, and synovial membrane was performed.

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Materials and methods

Animals

This study was approved and conducted according to the guidelines of the Hebrew University Animal Healthcare Ethics Committee.

Male ICR (CD-1) mice 8–10-weeks-old (Harlan, Israel) were used. The mice were held in specific pathogen free (SPF) conditions at the Hebrew University Ein Kerem animal facility and were given access to conventional chow and tap water *ad libitum*.

Materials

Recombinant murine VEGF165 (PeproTec, Rocky hill, NJ, USA) was dissolved in physiological saline (Teva, Israel) to reach a concentration of 0.05 mg/μl.

Experimental design

The experimental group consisted mice that received intra-articular injections, to the synovial space (see injection procedure below), of 20 μl VEGF165 solution once a week over a period of 4 weeks. Mice were sacrificed 1,2,3,4,6 and 8 weeks post-first injection, 10 mice per time point. The effect of VEGF on OA development was examined by histological evaluation. In the control groups, 10 mice were not subjected to any kind of treatment (sham group) and 20 mice received one intra-articular injection of 20 μl of physiological saline (0.9% NaCl, Pharmaceutics Department, Hadassah Medical Center, Jerusalem, Israel) and were sacrificed 2 and 8 weeks after injection, 10 mice at each time point.

VEGF administration

Mice were anesthetized using a 0.15 μl Xylazine 2% and 0.85 μl Ketamine HCl injected intra-peritoneally (i.p.). Then the left rear knee of each mouse was shaved and the patellar ligament was exposed to allow an approach from the lateral side of the knee, using a syringe equipped with a 27 gauge needle. The needle was inserted beneath the patellar ligament, into the intra-articular space and a volume of 20 μl solution containing either saline, or VEGF was introduced.

Tissue analysis

At the above-described time points, mice were sacrificed with an overdose of the anesthesia solution injected i.p. Their rear left (injected) and right (non-injected) knees were removed, fixed in buffered formaldehyde, decalcified with a calcium-chelating agent (Calci-Clear Rapid, National Diagnostics, Atlanta, Georgia, USA), and embedded in paraffin. Sections, 6 μm thick, were cut and stained with either Hematoxylin & Eosin (H&E) or Safranin-O, according to the standard protocols (see below). The lateral and medial aspects of both the distal femoral bones and the proximal tibial bones, separated by the cruciate ligament, were examined separately. Pathological changes observed in each of the four aspects of the knee joint were graded, according to a score shown in Table I, based on the Mankin score, and modified to include bone sclerosis.

Table I
Histological scoring

	Finding	Grade
Structure	Normal	0
	Slight surface irregularity	1
	Fibrillation reaching zones 1–2	2
	Fibrillation reaching zone 3	3
	Fibrillation reaching zone 4	4
	Cracks (near the tidemark)	5
	Erosion of zones 1–3	6
	Erosion of zone 4	7
	Exposed bone	8
Cluster appearance	Normal	0
	Cluster appearance	2
Tidemark	Intact	0
	Multiple	1
	Indistinct	2
	Crossed by blood vessels	3
	None	0
Osteophyte formation	Cartilage increment	1
	Early osteophytes	2
	Developed osteophytes	4
Subchondral bone	Normal	0
	Sclerosis	1
Synovial membrane	Normal	0
	Hyperplasia – mild	1
	Hyperplasia involving half the synovial space	2
	Hyperplasia involving the entire synovial space	3
Safranin-O staining	Strong staining of the cartilage	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No stain	4

Imunohistochemical staining of VEGF

Imunohistochemical staining of VEGF was performed on three representative knees, with scores typical for their sacrifice date, selected from each of the control and VEGF groups. Imunohistochemical staining was performed on formalin-fixed, paraffin-embedded bone sections, using the avidin–biotin–peroxidase complex method using horseradish peroxidase (HRPO)-labeled anti-mouse immuno-globulin (Histofine®Simple Stain Souse MAX-PO, Nichirei Biosciences Inc., Tokyo, Japan) and Diaminobenzidine substrate (SIGMA FAST 3,3′-Diaminobenzidine tablets, Sigma, Rehovot, Israel). Non-specific staining was avoided using incubation in 30% H_2O_2 to deactivate endogenous peroxidase and using M.O.MT.M kit (Vector Inc. Burlingame, CA, USA) according to the manufacturer instructions. VEGF staining was performed using monoclonal murine anti-human VEGF antibody (1:25 dilution, DAKO, Copenhagen, Denmark), which recognizes murine VEGF.

Antigen retrieval was performed by heat treatment of 88°C overnight before deparaffinization and incubation for 30 min in 1 μM EDTA (pH 8) after deparaffinization. Slides were counter-stained with Mayer's Hematoxylin. As a control, the same procedure was performed on a sequential section omitting the anti-VEGF antibody.

Histo-morphometric analysis

Chondrocytes number was assessed by histo-morphometry. The stained slides were analyzed using a computerized morphometric system (WinScanArray3; Galai, Migdal Haemek, Israel) connected to a light microscope (BH-2; Olympus, Tokyo, Japan). In each slide, consecutive microscope fields covering the entire articular cartilage of the examined knee were acquired (at magnification 400× and resolution of 1024 × 1024) by a color video camera (DXC-151AD; Sony, Tokyo, Japan). After acquisition, the images underwent automated light analysis and noise removal procedures to ensure color and image quality standardization in all analyses. Using PC-based Image analysis software (ImagePro+v4.5, Media Cybernetics, USA) the total area (in μm^2) of the cartilage was measured by

manually tracing the borders of the articular cartilage, and the chondrocytes were enumerated by assigning a color threshold using RGB color separation to isolate the chondrocytes nuclei, as previously described. The isolated nuclei were counted automatically by the "ImagePro" software.

For each image, ratios of chondrocytes number:cartilage area, giving the number of chondrocytes per 1 μm cartilage, were calculated using Excel software.

Statistical analysis

Statistical evaluation was performed using SPSS software, version 12.

The Kruskal–Wallis nonparametric test was used to compare more than two groups of variables. Significance was set at $P \leq 0.05$. When differences were found, the Mann–Whitney nonparametric test was used to confirm significant differences between two groups of variables, using the Bonferroni correction to set *P*-values.

Results

Progressive osteoarthritic changes were noted in all the joint tissues of the VEGF-treated animals, portraying a sequence of OA progression.

Tidemark multiplication

Changes in tidemark appearance are shown in Fig. 1(A) as mean number ± SE. Tidemark alterations, indicating cartilage calcification, began 1 week after VEGF injection in nine (out of 10) knees. By the 4th week [Fig. 1(B)], tidemark multiplications were observed in almost all aspects of the knee in seven (out of eight) knees. This is opposed to both control groups, in which only a single tidemark or occasionally two tidemarks were noticed. The appearance of such a limited number of tidemarks in the control groups is considered normal.

Subchondral bone sclerosis

Marked sclerosis was observed in the early weeks of the experiment and is presented in Fig. 1(C) as mean score ± SE. In the 2nd and 3rd weeks following VEGF injections sclerosis was observed in eight (out of 10) and 10 (out of 10) knees, respectively, involving the medial and lateral sides of both tibias and femurs. The frequency and severity of subchondral sclerosis declined 21 days post the VEGF injection. The disappearance of the sclerosis suggests that the subchondral bone has the potential to remodel once the cause (i.e., VEGF) is eliminated.

At week 4, none of the mice demonstrated sclerosis; and at that time VEGF was detected in the endothelial lining of the sinuses and in osteoblasts of the trabecular bone [Fig. 4(D)]. By week 6, subchondral sclerosis was detected in only three (out of eight) knees. Sclerosis, indicated by slight diminution of the marrow space, was rarely observed in the control groups [Fig. 1(C)].

Osteophyte formation

In this study osteophyte formation was rarely observed. In mice receiving weekly VEGF injections, osteophytes were observed only

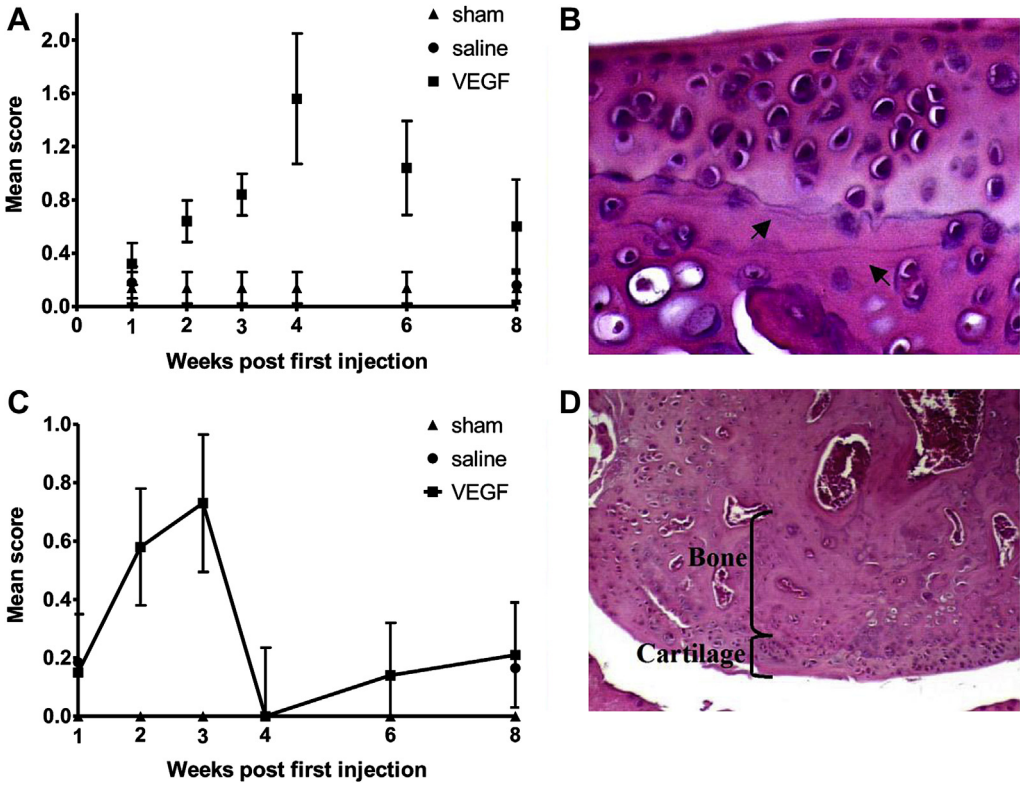

Fig. 1. Changes in the calcified tissues following VEGF injections. Following VEGF injections alterations in the tidemark (A), and in bone sclerosis (C), were evident. Histological section of: (B) right tibial condyle of VEGF injected knee after 4 weeks showing multiple tidemarks (arrows), and of the (D) right femoral condyle of VEGF injected knee showing bone sclerosis beneath a thinned layer of articular cartilage.
Values are of mean scores ± 95% confidence interval. For scoring values see Table I. *A: $P = 0.0054$; B: $P = 0.042$.

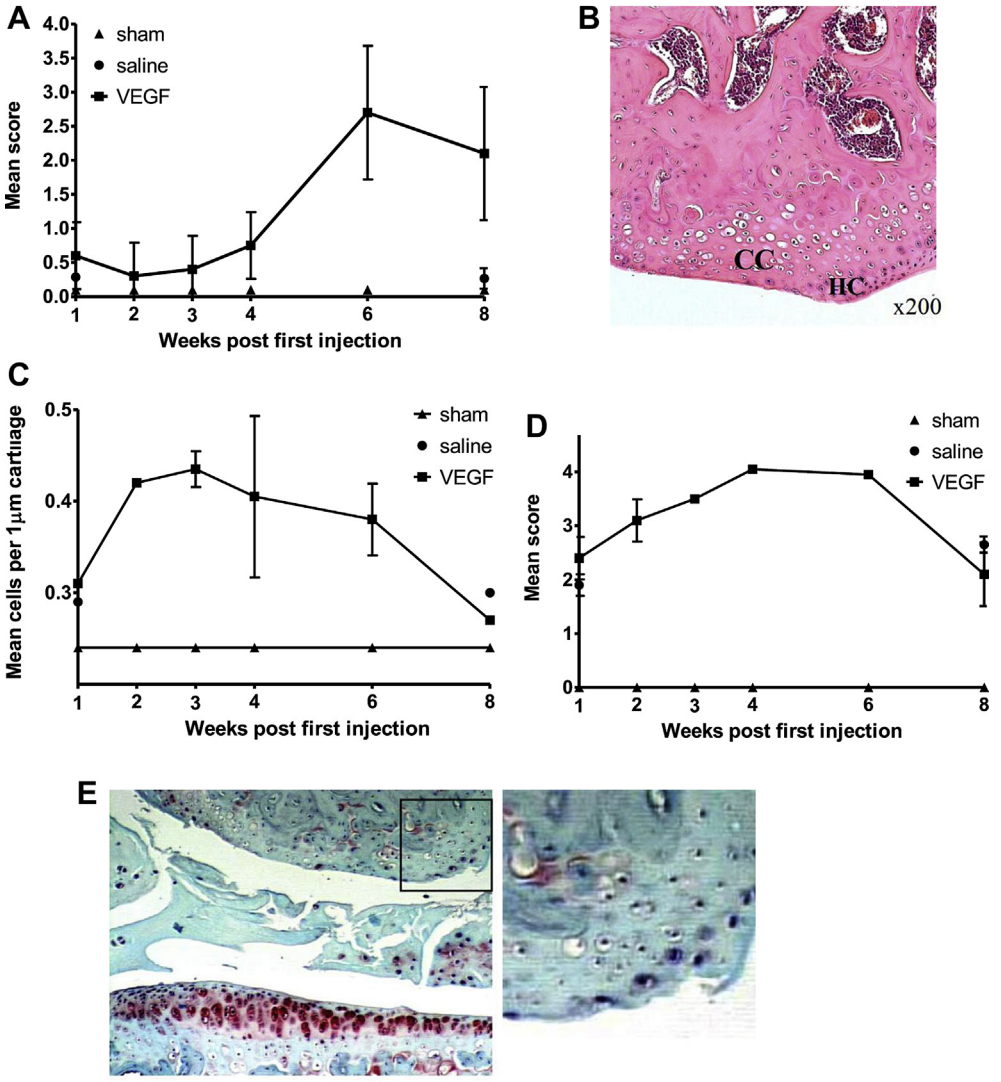

Fig. 2. Cartilage alterations following VEGF injections. (A) Cartilage morphological changes over 8 weeks. (B) Erosion of the articular cartilage, after 8 weeks, in a VEGF injected left femoral condyle. (C) Mean cell number per 1 μm cartilage ± SE, presenting cellularity rate over 8 weeks of the experiment. (D) Safranin-O stain score over 8 weeks, where higher score designates loss of stain. (E) Left femoral condyle of VEGF injected knee after 6 weeks stained for Safranin-O presenting loss of stain in the cartilage. The area in the rectangle is enlarged on the right showing loss of staining where the articular cartilage is left. Values are of mean scores ± 95% confidence interval.
For scoring values see Table I. A: $P = 0.039$; C: $P = 0.0047$; D: $P = 0.0002$. HC, hyaline cartilage; CC, calcified cartilage.

8 weeks after initiation of treatment. In the control groups no osteophytes were found.

Hyaline cartilage degradation

In both sham- and saline-injected control groups cartilage was generally intact; occasionally mild surface irregularities were noticed.

On the other hand, the cartilage of mice receiving VEGF injections on a weekly basis underwent progressive degradation, which was most severe at the 6th and 8th weeks [Fig. 2(A) and (B)]. Between the 1st and 4th weeks after the first injection, no alterations or mild irregularities (grade 1) were found in the injected knees. Interestingly, during the 3rd week, VEGF was observed in chondrocytes in the superficial layer of the articular cartilage [Fig. 4(C)]. However, 6 weeks after initiation of treatment (2 weeks after the last injection), six out of eight knees demonstrated severe erosion, and at week 8, five (out of eight) knees had extensive fibrillation and erosion.

Cartilage cellularity

Changes in cartilage cellularity, measured by histo-morphometry, are shown in Fig. 2(C) as mean number ± SE of cartilage cellularity ratio.

Cartilage cellularity ratios measured for the sham- and saline-injected control groups were 0.25 and 0.3 cells per 1 μm cartilage, respectively. In mice receiving weekly VEGF injections, 2 or 3 weeks after the onset of treatment chondrocytic ratios were significantly elevated (ratio of 0.41 and 0.42 cells per 1 μm cartilage, respectively). However, chondrocytic numbers declined to baseline levels by week 8.

Cartilage mucopolysaccharide loss

Gradual decrease in Safranin-O staining, indicating mucopolysaccharide loss, was observed in the cartilage of treated knee joints [Fig. 2(D)]. Knee joints from sham controls showed no alterations in mucopolysaccharide content. The saline injected knees showed

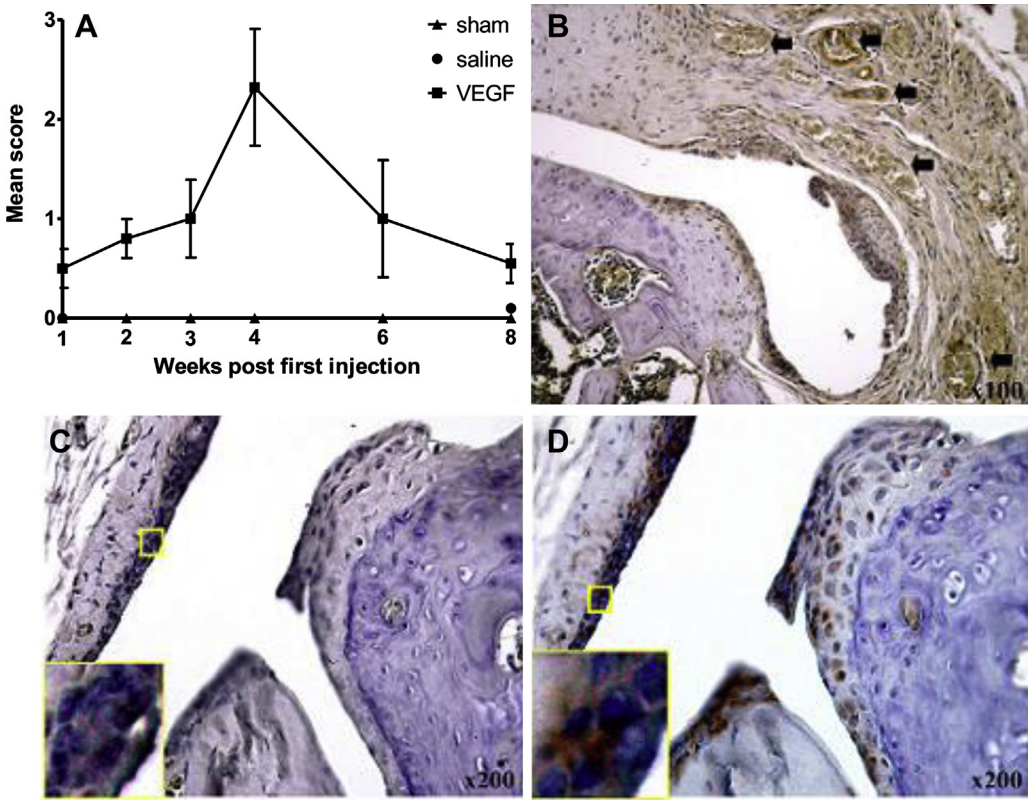

Fig. 3. Synovial alterations following VEGF injections. (A) Synovial changes over 8 weeks. (B) VEGF immunostaining of synovial membrane near the left tibial condyle in VEGF injected knee after 4 weeks showing vascular hypertrophy and staining of large blood vessels for VEGF. (C) Control (secondary antibody only) and (D) Immunostaining of VEGF of the synovial membrane of a right tibial condyle after 3 weeks, showing marked cytoplasmatic staining of synoviocytes (rectangle enlarged at the bottom) in the stained specimen. Values are of mean scores ± 95% interval.
For scoring values see Table I. A: $^{*}P = 0.013$.

mildly reduced Safranin-O staining at week 8. Progressive loss of mucopolysaccharides was observed after weekly injections of VEGF.

By weeks 4 and 6, complete mucopolysaccharide loss was observed in all specimens [Fig. 2(D) and (E)].

Synovial membrane hyperplasia

Synovial membrane hyperplasia, detected on H&E stained sections, increased after VEGF injections and declined over time when VEGF was not injected.

These changes are presented in Fig. 3(A) as mean score +SE. Synovial membrane hyperplasia was accompanied by the appearance of prominent blood vessels in the joint capsule [Fig. 3(B)]. In both control groups, the synovial membrane consisted of 1–2 cells thick cellular layer.

Following weekly injections of VEGF, synovial hyperplasia was observed at weeks 2 and 3 after the primary injection in eight (out of 10) and nine (out of 10) knees, respectively. Of these, six (out of eight) and five (out of nine) knees showed synovial hyperplasia in both medial and lateral aspects. At this time frame, VEGF was detected by immune-histochemistry in the synoviocytes [Fig. 3(C) and (D)]. By week 4 after initiation of treatment all knees examined showed synovial hyperplastic changes in all joint aspects.

Cessation of VEGF injections on week 4 reduced synovial hyperplasia, reaching a level of five/eight knees on week 8. In these weeks (i.e., 6 and 8 after treatment initiation), VEGF staining was confined to synoviocytes and was not observed in other tissues.

Interestingly, the opposing (non-injected) knees of animals treated by VEGF presented similar changes and arthrosis progression pattern in cartilage and bone. However, these changes were less frequent and less severe in comparison to the injected knee.

VEGF expression in osteoarthritic knees

The presence of VEGF in knees developing OA was detected by specific immunostaining. In the healthy mouse VEGF staining was not detected in the cartilage [Fig. 4(A)], bone [Fig. 4(B)] and synovium [Fig. 1(C)].

Following VEGF injections, VEGF staining first appeared after 3 weeks. It was observed in the cytoplasm of synoviocytes [Fig. 3(D)] and in chondrocytes in the superficial layer of the articular cartilage [Fig. 4(C)]. At that time, VEGF staining was also observed in the endothelial lining of the sinuses and in osteoblasts in the trabecular bone [Fig. 4(D)]. A similar staining pattern was observed in mice sacrificed at week 4. By weeks 6 and 8 after treatment initiation, VEGF staining was confined to synoviocytes and was not observed in other tissues.

Discussion

The present study revealed that exogenous VEGF, injected intra-articularly in healthy mice, initiated a full-range osteoarthritic process in the knee joint.

The early signs of OA were synovial hyperplasia associated with an increase in blood vessels quantities. Synovial hyperplasia and increased vasculature appeared concomitantly with both progressive calcification in the calcified layer of the articular cartilage, manifested by tidemark advancement, as well as by subchondral

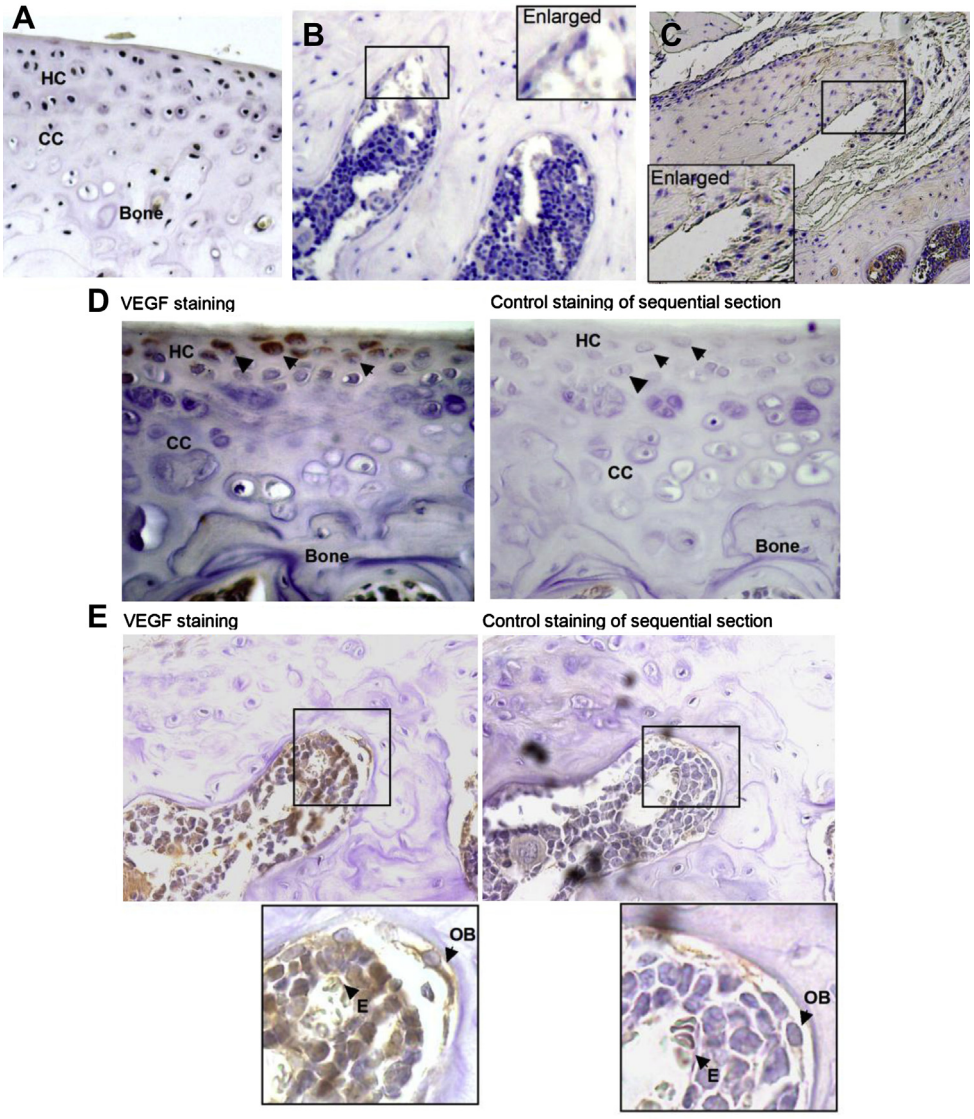

Fig. 4. Immunostaining of the cartilage and bone of VEGF injected knees. (A) Knee of Sham mouse shows no VEGF staining in the cartilage or (B) cells of the marrow or (C) in the synovium. (D) After 3 weeks of VEGF injection, chondrocytes of the hyaline cartilage in the injected knees show staining with VEGF (arrows). (D) Osteoblasts and endothelial lining of the sinuses of the injected knees show staining with VEGF, after 4 weeks of VEGF injection (areas in the rectangle are enlarged on the black marked figures at the bottom). HC, hyaline cartilage; CC, Calcified cartilage; OB, osteoblast; E, endothelial cell.

bone sclerosis. The tidemark represents the mineralization front of the cartilage and extension or multiplication of the tidemark displays a response to injury. These changes were accompanied by the expression of the effect of VEGF on synoviocytes, osteoblasts, and endothelial cells in the marrow, and in the third week post-VEGF administration, on chondrocytes.

On week 6, following ossification and remodeling of the subchondral tissue, synovial hyperplastic changes reached their peak and cartilage degradation became more severe, with deep fibrillation, erosion of the cartilage, and cracking near the tidemark.

The sequence of events leading to OA following VEGF administration shows that the articular cartilage degradation occurred subsequent to alterations in the synovial and calcified layers. Subchondral bone supports a large portion of the load exerted on the joint, relative to the overlying hyaline articular cartilage[18]. Increasing rigidity of the subchondral tissues reduces absorbing capacity, thus exposing the cartilage to higher loads that contribute to its degradation[1]. VEGF seems to promote calcified cartilage ossification and subchondral bone remodeling and, as noticed in the later weeks of the experiment, may play a role in the pathogenesis of OA.

The expression of VEGF in chondrocytes in the superficial and mid zones of the hyaline cartilage of the VEGF-injected mice contrary to the controls suggests a further role for VEGF in the initiation and progression of OA.

Similar morphologic changes, to a lesser extent and severity, were observed in the contra lateral non-injected knees. It is possible that VEGF is absorbed into the circulation giving rise to systemic effects. Discontinuation of VEGF administration resulted in resolution of subchondral bone sclerosis and synovial hyperplasia. This suggests that consistent VEGF presence in these sites is required to maintain the alterations. Without VEGF, the possibility of spontaneous rehabilitation should be considered. The discordant healing of bone, despite marked cartilage degradation in this model, may be explained by the lesser ability of cartilage to regenerate relative to bone. Thus, injuries to the cartilage are

sustained for a longer period of time. The development of subchondral bone changes before the onset of the degenerative changes in the articular cartilage suggests the importance of subchondral calcifying tissues in promoting the progression of the disease.

The notion that impaired angiogenesis and vascular pathology is associated with OA, has been suggested previously. This study points to a probable role for VEGF in initiating and propagating OA firstly, by activating the synovial and calcified tissues of the joint. It is possible that VEGF affects the calcified tissues initially by altering subchondral tissue metabolism and promoting release of factors of vascular remodeling. These findings suggest that controlling VEGF secretion may help to prevent OA escalation and OA-associated joint deterioration.

Author contributions

All authors contributed equally to this work.

All authors are qualified for authorship in all aspects namely: Conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, provision of study materials or patients, statistical expertise, obtaining of funding, administrative, technical, or logistic support, collection and assembly of data.

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Conflict of interest

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