Cyclic strain influences the expression of the vascular endothelial growth factor (VEGF) and the hypoxia inducible factor 1 alpha (HIF-1α) in tendon fibroblasts

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Abstract

Neovascularization is involved in beneficial and detrimental processes of tendon pathology.

We investigated the influence of repetitive motion on the expression of the most important angiogenic factor, the vascular endothelial growth factor (VEGF) in the 3T3 NIH fibroblast cell line and in cultures of rat Achilles tendon fibroblasts. Monolayers of subconfluently grown cells were stretched in rectangular silicone dishes with cyclic uniaxial movement. Strain was applied over 24 h varying the frequency (0.5 - 1 Hz).

Fibroblasts (3T3 fibroblasts and rat Achilles tendon cultures) cultivated without the application of cyclic strain released measurable VEGF amounts into their culture supernatants. Cyclic stretching of the cells with a frequency of 1 Hz resulted in an increased expression of VEGF. A low frequency (0.5 Hz) reduced VEGF expression to control levels. RT PCR revealed VEGF 121 and VEGF 165 as the only splice forms that were induced by cyclic stretching. Western blot experiments could further show that cyclic stretching induced activation of the transcription factor HIF-1α. These results demonstrate that mechanical factors are involved in the regulation of VEGF expression in tendon tissue.

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Introduction

Neangiogenesis might be involved in beneficial but also in detrimental effects to tendon tissue. Revascularization is an important process for the remodeling of autologous tendon grafts or for the restoration of the vascular pattern during tendon healing [32,37]. On the other hand, in degenerative tendon disorders histopathologic findings and experimental findings revealed increased vascularity and increased blood flow [8,20,23].

Degenerative changes weaken the stability of the tissue and therefore predispose a tendon for spontaneous rupture [20,23,27].

Angiogenesis is controlled by various mitogenic, chemotactic, or inhibitory peptides and lipid factors that act on invading endothelial and smooth muscle cells [16]. One of the most important angiogenic factors is the vascular endothelial cell growth factor (VEGF) or vascular permeability factor (VPF), a glycosylated protein of 46 48 kDa composed of two disulfide-linked subunits which originally was isolated from tumor cells [45].

In previous studies the presence of the angiogenic peptide VEGF was determined in fetal human Achilles tendons, in autologous tendon grafts during the remodeling process, in healing tendons but also in biopsies of patients with degenerative changes of the Achilles tendon [11,13,33,34,36,38,40]. Angiogenesis contributes to the repair and remodeling of the injured tendon, but
also may weaken its mechanical stability by proteolysis of the extracellular matrix (ECM) by the invading endothelial cells [36]. Secretion of metalloproteinases (MMPs) facilitates capillary growth via ECM dissolution [15]. A recent study using a 3D in vitro angiogenesis model has shown that angiogenesis alters the material properties of the ECM [26]. This mechanism could be causative for the limited mechanical strength of autologous tendon grafts used for anterior cruciate ligament (ACL) reconstruction or for the pathogenesis of tendon degeneration. The rapid deprivation in ultimate strength of ACL grafts is accompanied by a strong upregulation of VEGF and angiogenesis [37,51]. In degenerative tendons, tissue neovascularization was accompanied by high VEGF levels [38]. The VEGF induced upregulation of MMPs might contribute to the tissue damage during the degenerative tendon disease.

Previous studies have shown that hypoxia and several growth factors upregulate VEGF expression synergistically in tendon tissue [16,21,35]. Both factors might be responsible for the increased VEGF levels observed during the remodeling of autologous tendon grafts and in degenerative tissue [34,37,38]. However, during daily living or sporting activities tendon tissue is subjected to repetitive loading and the consecutive changes in mechanical environment can significantly affect the mechanical properties of tendon tissue [52]. While cyclic loading is known to be beneficial to tendon health [18] repetitive tissue strain has also been implicated in the etiology of overuse injuries of tendons [4]. In tendon cells cyclic strain activates a wide array of cellular machinery, including DNA synthesis, mitosis and cell differentiation [1,3,6,9,10]. In a previous study we could show that in tendon cells VEGF expression is sensitive to the application of intermittent hydrostatic pressure [33]. Intermittent hydrostatic pressure decreased VEGF expression of rat Achilles tendon fibroblasts significantly [33].

The aim of the current study was to examine the effect of cyclic loading on VEGF expression in tendon cells. Since the transcription of the VEGF gene is mediated by the hypoxia-inducible factor-1 (HIF-1) [28,43,56] and since a recent study has shown that HIF-1 (the limiting protein for the formation of the dimeric transcription factor) is upregulated in the non-ischemic but mechanically stressed myocardium [25] we further evaluated the induction of HIF-1 expression in tendon cells under mechanical stress.

Materials and methods

Cell culture of rat tendon fibroblasts and 3T3 fibroblasts:

For cell culture experiments 3T3 fibroblasts and cultures of rat Achilles tendon cells were used. Achilles tendons were dissected from postnatal (2-5 days old) rats, cut into small pieces, transferred into a small volume (2 ml per 10 cm² culture flask; Tissue Culture, Switzerland) of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), and cultured for 24 h at 37 °C. Then, further 3 ml DMEM plus 10% FCS were added, and tissue pieces left for another 24 h at 37 °C. During this time, fibroblasts migrate out of the tissue and adhere to the bottom of the culture dish. After removing medium and the remaining floating tissue pieces, fresh DMEM plus 10% FCS was added, and cells cultivated for 96 h at 37 °C. For subculture, cells were detached by trypsin (0.1%, 1:250, Gibco, Paisley, UK) treatment, 10% were seeded onto rectangular silicon dishes which have been coated with type I collagen and cultivated for 24 h in DMEM plus 10% FCS. Then, medium was replaced by DMEM (without FCS).

Both cell types have been used for the VEGF immunostaining, the VEGF ELISA measurements, the VEGF Western blot and the RT-PCR analysis. For the HIF-1α Western blot only rat Achilles tendon fibroblasts have been used.

Cyclic strain application

For the application of uniaxial strains, the dishes with the monolayer cell cultures were subjected to cyclic, homogenous stretching in a custom made six station motor driven apparatus. The eccentric motor allowed variation of stretch magnitude (0.5-10%) and stretch frequency (0.5-5 Hz). The dishes were optically clear and had a cell culture surface area of 50 mm x 23 mm. This model has been used in previous studies [12,46,47,49,54]. The dishes were molded of two component silicone elastomer containing Siliconeel RTV 270 and crosslinker A 47 (Rhone Poulenc, 1 übeck, Germany). The suitability of the dishes for cell cultures has been described elsewhere [12,46,47,49,54].

The dishes were then cyclically (sinusoidally) stretched with 8% uniaxial strain at different frequencies (0.5, 1 Hz) for 24 h. The type I collagen coated silicon dishes have been placed in the stimulation apparatus and cultivated for 24 h in DMEM plus 10% FCS. Each experiment has been repeated 5 times, each with a different culture. In each experiment we used 6 stimulated and 6 unstimulated culture wells for the analysis of VEGF and HIF-1α expression.

Enzyme-linked immunosorbent assay (ELISA)

For ELISA, the supernatant of the cell cultures was homogenized in 150 mM NaCl 20 mM Tris-HCl-buffer, pH 7.4; a soluble fraction obtained by centrifugation (48 000 x g, 60 min), and aliquots (100 µl) were analyzed by a sandwich ELISA (R&D Systems, Minneapolis, MN, USA) that detects all VEGF splice forms. Mouse recombinant VEGFα (PreproTech, Rocky Hill, NJ, USA) served as standard.

Western blots

For Western blots, samples were reduced in the presence of 10 mM dithiothreitol, proteins separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% gels), transferred onto nitrocellulose membranes that were blocked and incubated with antibodies according to standard techniques as described [38,39]. Signals were detected by chemiluminescence reaction (ECL-Plus: Amersham-Pharmacia, Uppsala, Sweden). The films were scanned with a laser film digitizer, and VEGF or HIF-1α expression determined by optical density measurement using the image analysis system PC Bas 2.0 (ray test; Isotopenmessgeräte GmbH). For HIF-1α only the rat Achilles tendon fibroblasts have been used.

Immunohistochemical analysis

For immunohistochemical analysis, cultures were fixed in 3% paraformaldehyde, embedded in paraffin, irradiated at 70 °C in a microwave oven with 5% hydrogen peroxide in 0.01 M sodium citrate buffer, pH 6.0 (twice for 5 min), immersed in 5% formaldehyde and rinsed in Tris buffered saline solution, the sections were incubated with biotinylated goat anti-rabbit immunoglobulins (1:200 in Tris Base Buffer, 45 min; Dako, Glostrup, Denmark), washed in...
Tris buffered saline solution for 5 min and incubated with streptavidin-peroxidase (1:50, 30 min; StrepAB Complex/HRP, DAKO, Glostrup, Denmark). The substrate was ABC (DAKO, Glostrup, Denmark). Negative controls were incubated with the secondary antibody and the Strept AB complex alone. Nuclei were counterstained with hemalum (2,3,5-s), washed in distilled water, and finally covered with Aqua Tes (Merck, Darmstadt, Germany).

Reverse transcription polymerase chain reaction (RT-PCR) for VEGF splice variants

For RT-PCR, the pellets of the cell cultures were used. RNA isolated by the phenol-guanidinium thiocyanate method, purified by isopropanol and repeated ethanol precipitation, and containing DNA was destroyed by digestion with RNase-free DNase I (20 min 25 °C; Boehringer, Mannheim, Germany). After inactivation the DNase (15 min 65 °C), cDNA was generated with 1 µl (20 pmol) oligo(dT)₅ primer (Amersham Pharmacia Biotech, Uppsala, Sweden) and 0.8 µl superscript RNase H reverse transcriptase (Gibco, Paisley, UK) for 60 min at 37 °C as described [7]. For PCR, 4 µl cDNA were incubated with 30.5 µl water, 4 µl 25 mM MgCl₂, 1 µl dNTP, 5 µl 10x PCR buffer, and 0.5 µl (2.5 U) Platinum Taq DNA polymerase (Gibco) and the following primers (2.5 µl each containing 10 pmol; selective for VEGF splice variants 5'-GGG GGC TTC CTC GCA GTG-3' (sense) and 5'-TCA CCG CCT TGG CTT GTC AC-3' (antisense) yielding different bp fragments (30 cycles, annealing 7; 94 °C 1; 65 °C 1; 72 °C 1; 30 s).

Statistics

For statistical analysis of the ELISA results and the OD measurements the unpaired Mann Whitney U Wilcoxon test have been used. The p-level for significance was set at 0.05.

Results

VEGF immunostaining

Both 3T3 fibroblasts and rat Achilles tendon fibroblasts which have been cultivated under uniaxial strain at a frequency of 0.5 and 1 Hz showed a longitudinal cell shape and a parallel orientation. Cells under the influence of cyclic stretching at a frequency of 1 Hz could be intensively stained with the VEGF antibody. Cells cultivated under cyclic stretching of 0.5 Hz as well as randomly orientated control cells showed only a weak immunoreaction (Fig. 1).

VEGF Western blot

The ELISA results could also be verified in Western blotting experiments which further prove the specificity of the immunoreaction. Two bands of 45 kDa (dimer) and 22.5 kDa (monomer) that correspond to the highly glycosylated VEGF protein were heavily stained in cultures which has been subjected to uniaxial strain (Fig. 3). The strongest bands have been observed in cells that have been stretched with a rate of 1 Hz. For rat Achilles tenocytes the mean optical density of the 1 Hz group (OD: 506.732; ±393.36) was significantly higher than in the 0.5 Hz (OD: 2426.56; ±218.95) and unstimulated group (OD: 152.43; ±23.62). For 3T3 fibroblasts there was also a significant difference between all three groups.
(1 Hz group: OD: 3940.67; ±683.69; 0.5 Hz group: OD: 2357.32; ±368.35; unstimulated group: OD: 202.89; ±31.63) (p < 0.05).

RT-PCR

Since the VEGF antibody detects all splice forms and SDS-PAGE lacks resolution to separate variants with 20-40 residues differences due to the high glycosylation of the VEGF protein, we analyzed the expression of different splice forms by RT-PCR with primers yielding differently sized products (Fig. 4).

From all cultures—3T3 fibroblasts (not shown) and rat Achilles tenocytes—two PCR products were obtained: one with 512 bp corresponding to VEGF120 and one with 644 bp corresponding to VEGF164. Both splice products were also obtained with other VEGF-positive tissues (fetal tendon tissue) whereas a cartilage from the growth plate yielded the splice forms VEGF120 and VEGF164 (716 bp; not shown) illustrating that the method has the resolution necessary to detect other forms. The strongest bands have been observed in cells that have been stretched with a rate of 1 Hz.

HIF-1α induction measured by Western blotting experiments

Western blotting experiments showed that the Achilles tendon cells subjected to uniaxial strain at 1 Hz expressed the hypoxia-inducible factor-1 (HIF-1α). A band of 103 kDa that corresponds to HIF-1α was heavily stained in cultures which have been subjected to uniaxial strain but not in unstimulated control cultures (Fig. 5). There was a significant difference in the OD between stretched cells (OD: 9387.48; ±778.28) and unstretched cells (OD: 2034.68; ±247.37) (p < 0.05).

Discussion

The ability of connective tissue to functionally adapt to the mechanically environment depends on the property of the cells to model and remodel tissue architecture
under mechanical strain. The responsiveness of connective tissue cells such as osteoblasts, chondrocytes and fibroblasts to mechanical strain has been confirmed by several in vitro investigations and the effect of cyclic strain on tendon cells has been associated with a diverse array of biological responses [1 3,6,5,12,19,24,30,38,46, 47,49,53,54]. These cellular features are necessary to restore the normal mechanical properties of the tissue after injury or during a remodeling process (e.g. remodeling of autologous tendon grafts after ACL reconstruction).

Due to various in vitro studies the evidence that stretch is a stimulus for growth factors has emerged. The results of the current study demonstrate that mechanical stretch regulates the expression of the vascular endothelial growth factor (VEGF) in tendon cells. We used a loading system utilizing controlled uniaxial distension of a deformable culture dish. Many groups have used different longitudinal stretch systems to study the effect of mechanical stress on cells [24,31,32,46,47,49,54]. Since the local strain distribution on the culture surface depends strongly on the design of the culture dish it is difficult to compare results between groups. We used a silicon well with dimensions as first stated by Zeichen et al. [54]. Since this group studied the effect of mechanical strain on the expression of various proteins in tendon cells [12,46,47,49,50,52] we used the same model to study the effect of mechanical strain on VEGF expression.

In this study a moderate stretch length was chosen to reduce the risk of altering cell morphology [31]. Neidlinger Wilke et al. [31] have shown that fibroblasts have an optimal alignment response at a stretch rate of 8%. A potential disadvantage of our cell culture model is that the local strain distribution on the culture surface has not been evaluated. Culture surface strains within the wells might be heterogeneous.

Evidence that VEGF is upregulated by stretch of the ventricular wall was firstly provided by Li et al. [29]. They found a marked increase in VEGF mRNA after diabetic pressure had been increased and further showed that this increase was mediated, at least in part, by tumor growth factor-beta (TGF-beta).

Previous in vitro studies have examined the effect of mechanical stretch on VEGF expression in other cell types than tendon fibroblasts [14,44,48]. In renal mesangial cells, stretch increased VEGF mRNA expression 2.4-fold, peaking at 6 h [17]. In cardiac myocytes, a 1.8-fold increase in VEGF mRNA that peaked at 2 h was found [55]. In a pulmonary cell culture model [41], stretch increased VEGF mRNA expression (1.8-fold). In contrast, intermittent hydrostatic pressure decreased VEGF expression in cultures of rat Achilles tendon fibroblasts [33].

The present study shows that the effect of uniaxial stretching was highly sensitive to strain frequency since strain rates of 1 Hz resulted in increased VEGF levels. At 0.5 Hz VEGF concentrations measured with the ELISA differed not significantly from the levels of the control cultures. In contrast the OD measurement of the Western blot revealed a significant difference between 0.5 and 1 Hz group. This disagreement might be a result of a different sensitivity of the methods. On the other hand, it is well known that mechanical stimulation parameter such as the frequency strongly affect cellular reactions [24]. Most studies investigating the effect of uniaxial strain on cells use a frequency of 1 Hz because these conditions are postulated to be physiological [24].

Disadvantages of in vitro loading include the use of artificially constructed environment that may be lacking essential mediators or cytokines, the application of arbitrary load cycles, and heterogenous cell culture surface strains. Although an “in vitro overload situation” is difficult to define, the results of this study indicate that an unphysiological loading time (24 h) increases VEGF expression only at higher strain frequency. These observations support previous in vivo findings which have shown that VEGF and its receptors VEGFR-1 and VEGFR-2 are strongly expressed in biopsies of patients with overload injuries of the Achilles tendon [36].

Previous studies have shown that VEGF is not only re-expressed in tendon overuse injuries but also in a tendonotomy model in sheep [34] and during the remodeling of autologous tendon grafts [37]. In contrast to overload injuries both situations are characterized by a limited activity. Therefore other factors than mechanical factors might be responsible for VEGF re-expression under these conditions.

One of the most important regulators for VEGF gene expression in vivo and in vitro is oxygen tension. VEGF mRNA expression is induced by exposure to low \( pO_2 \) in a variety of normal and transformed cell types [16]. A 28-base sequence has been identified in the 5'-promoter of the rat and human VEGF gene which mediates hypoxia induced transcription [28]. Such sequence reveals high degree of homology and similar protein binding characteristics as the hypoxia-induced transcription factor-1 (HIF-1) binding site within the erythropoietin gene [28]. HIF-1 is a heterodimeric, helix-loops-helix protein consisting of two subunits HIF-1\( \alpha \) and HIF-1\( \beta \).

Gene knockout studies provided evidence for a critical role of HIF-1\( \alpha \) [21] which is the rate-limiting protein for the formation of the HIF-1 complex. Because HIF-1\( \alpha \) induction was initially described for conditions of decreased oxygen pressure, the majority of studies on HIF-1\( \alpha \) regulation have concerned hypoxic conditions. However, recent evidence suggests that HIF-1\( \alpha \) can also be induced under normoxic conditions by growth factors, hormones, or nitric oxide [25].

Kim et al. [25] were the first who studied the role of HIF-1\( \alpha \) for stress mediated VEGF induction in the rat heart. They found that HIF-1\( \alpha \) plays an important role.

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in the induction of VEGF in the non-ischemic and mechanically stretched myocardium, and that this is regulated by stretch-activated channels and the PI3K/Akt/FRAP pathway [25]. The cell culture model of the present study confirms the in vivo findings of Kim et al. [25] and demonstrates that HIF-1 protein is induced by cyclic strain in tendon cells. Since VEGF expression was increased only at a frequency of 1 Hz we investigated HIF-1 induction at this frequency.

In the present study, the role of TGF-β in VEGF induction has not been examined. On the basis of several previous reports [22,42], TGF-β could be another important factor responsible for VEGF induction. Li et al. [29] and Zheng et al. [55] demonstrated the involvement of TGF-β in the stretch-mediated induction of VEGF in cardiac myocytes.

In conclusion, this study demonstrates that mechanical stress induces HIF-1α and VEGF in tendon cells. VEGF-mediated angiogenesis is considered to have beneficial [34,37] but also detrimental effects on tendon tissue [36,38], either by increasing the regenerative potency, but also by weakening the tissue during this process.

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References


[34] Petersen W, Pufe T, Unterhauser F, Zantop T, Mentlein R. The splice variants 120 and 164 of the angiogenic peptide vascular endothelial cell growth factor (VEGF) are expressed during Achilles tendon healing. Arch Orthop Trauma Surg 2003; (Mar);15.


