The distribution of vascular endothelial growth factor in human meniscus and meniscal injury model

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ABSTRACT

Background

The meniscus plays an important role in controlling the complex biomechanics of knee. Meniscus injury is common in knee joint. A perimeniscal capillary plexus supplies the outer meniscus, whereas the inner meniscus is composed of avascular tissue. Angiogenesis factor, such as vascular endothelial growth factor (VEGF) has important roles in promoting vascularization of various tissues. VEGF-mediated neovascularization is beneficial to the healing of injured tissues. However, the distribution and angiogenic role of VEGF remain unclear in meniscus and injured meniscus. We hypothesized that VEGF can affect meniscus cells and modulate healing process of meniscus.

Methods

Meniscus was obtained from total knee arthroplasty patients. Meniscal injury was created by a microsurgical blade. VEGF expression was investigated by polymerase chain reaction and immunohistochemical analyses.

Results

PCR results showed that in meniscal tissue, VEGF expression was undetected, whereas VEGF expressions were found in injured meniscal tissue (outer > inner). Immunohistochemical analyses revealed that VEGF was mainly detected in outer and around injured areas of meniscus. However, VEGF concentration was similar between inner meniscus-derived conditioned medium and outer meniscus-derived medium.

Conclusions

This study demonstrated that both inner and outer regions of meniscus contained VEGF. VEGF deposition was highly detected in injured meniscus. Our results suggest that VEGF may be preserved in extracellular matrix as the healing stimulator of damaged meniscus, especially in outer meniscus.

Keywords: vascular endothelial growth factor (VEGF); meniscus; meniscal injury; hypoxia-inducible factor-1α (HIF-1α)

INTRODUCTION

The meniscus is a fibrocartilaginous tissue that plays an important role in controlling complex biomechanics of knee. Proper functioning of meniscus depends on the composition and organization of its extracellular matrix ECM)[1]. In adult human meniscus, only outer 10–25% of meniscus is vascular area, whereas inner 70–80% of the meniscus is composed of avascular tissue[2]. Cells in the vascularized area(red-red region) are more fibroblast-like in appearance, while cells in the middle(white-red region) and inner(white-white region) zones show more chondrocyte-like shapes. Based on these findings, several authors demonstrate that healing potential of outer meniscus is higher than that of inner meniscus[3]. According to the injured area and severity of meniscal injury, meniscal healing rate is between 67% and 92%[4]. Previous studies demonstrate that vascularization is a key factor on healing of injured meniscus, so outer meniscus has a better healing effect than inner meniscus[5].

Vascular endothelial growth factor(VEGF) is a major angiogenesis factor. VEGF can promote both physiology and pathological vascularization[6]. The effect of VEGF on vascular endothelial cells has been expanded to include migration and invasion into the basement membrane, proliferation, survival and the formation of fenestrations[7]. Several researchers have demonstrated that VEGF can help the healing of tissues, such as knee ligament[8] and cornea[9] by promoting vascularization. In rabbit, VEGF expression in avascular zone shows a significant increase 2 weeks after injury. This increased expression of VEGF may lead to healing of meniscal tear and be attributed to a possible increase in expression of antiangiogenic factors, notably endostatin[10]. However, the role and distribution of VEGF in human meniscus is unclear. In this study, we investigated the localization of VEGF in human meniscus and ex vivo meniscal injury model.

METHODS

Tissue, cells, and cell culture

Institutional Review Board approval and informed consent were obtained before all experimental studies. Macroscopically intact lateral menisci were obtained at total knee arthroplasty in patients suffering from osteoarthritis of the knee. Patients(four females, two males) were 69, 70, 72, 73, 76, and 78 years of age. Cases that maintained sufficient articular cartilage of lateral femoral condyle and undegenerated lateral meniscus in magnetic resonance imaging were selected. Meniscal tissues were divided into two groups: native meniscus and injured meniscus. In the native meniscus group, inner and outer meniscus cells were prepared from the meniscal samples as described[11, 12]. In brief, synovial/capsular tissues and the superficial zones of the menisci were removed carefully. The width of obtained lateral meniscus was 10–14 mm. The midpoint of meniscal width(5–7 mm in length from inner edge) was marked. Inner and outer meniscal tissues were prepared by careful cutting. Inner and outer meniscus cells were prepared by organ culture treatment. Attached cells(passage 0) were maintained with Dulbecco's modified Eagle's medium(DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum(FBS; HyClone, South Logan, UT), and 1% penicillin/streptomycin(Sigma). Meniscus cells between passages 1 and 2 were used.

In the injured meniscus group, the lesions were created with a microsurgical blade: a full-thickness, longitudinal, 5-mm-long lesions in both inner and outer meniscal area. After that, we divided the injured tissue into two groups: one group was for immunohistochemistry assay, and another group was separated into inner 2/3 and outer 1/3 meniscus for PCR and cells culture. 14 days later, we obtained the injured meniscal tissue from the first group to procced the immunohistochemistry assay. When 80% of the petri dish was covered with cells, we selected the suitable tissues to extract RNA for tissue RT-RNA assay. The cells were continued to culture. And the injured meniscus cells between passages 1 and 2 were used for RT-PCR.

RT-PCR and quantitative real-time PCR

RNA samples were obtained from superficial zone-excluded meniscal tissues and cultured meniscus cells. Total RNAs were isolated using ISOGEN reagent(Nippon Gene, Toyama, Japan). RNA samples(500 ng) were reverse-transcribed with ReverTra Ace(Toyobo, Osaka, Japan). The cDNAs underwent PCR amplification in the presence of specific primers using exTaq DNA polymerase(TaKaRa, Ohtsu, Japan). For all the RT-PCR fragments, the reaction was allowed to proceed for 32-36 cycles. The following specific primer sets were used: $VEGF_{exon3-4}$(5'-TGG TGG ACA TCT TCC AGG AG-3' and 5'-TTG GTG AGG TTT GAT CCG CA-3')[13, 14], HIF-1α(5'-CAC TTC CAC ATA ATG TGA GTT CGC-3' and 5'-GGT TCA CAA ATC AGC ACC AAG CAG G-3')[15], and glyceraldehyde- 3-phosphate dehydrogenase(G3PDH)[16]. Quantitative real-time PCR analyses were performed using a LightCycler ST-300 instrument(Roche Diagnostics, Mannheim, Germany) and FastStart DNA Master SYBR Green I kit(Roche Diagnostics) [17]. The cycle number crossing the signal threshold was selected in the linear part of the amplification curve. Amplification data of G3PDH were used for normalization. These assays were run in triplicate, and relative mRNA levels were normalized with the level of non-stretched inner meniscus cells for every sample.

Immunohistochemical assay

The deposition of VEGF in the whole meniscal tissues including the superficial zones was assessed by immunohistochemical analyses using a rabbit anti-VEGF(sc-152) antibody(working dilution 1:100 for overnight incubation, Santa Cruz, CA). An anti-VEGF antibody(sc-152), raised against a peptide mapping at the N-terminus of VEGF-A of human origin, recognizes the precursor and mature form of human VEGF(mapping to 6p21.1). A bovine serum albumin solution without the primary antibody was used as a negative control. Staining density of VEGF was quantified by Image J 1.31. Relative staining densities of VEGF and VEGF-positive cells count(/2500um^2) were assessed by mean values derived from five different images of meniscal bodies without the superficial zone of the menisci. Microscopic selection and evaluation of five different areas were performed by four observers.

Statistical analysis

All experiments were repeated in triplicate and similar results were obtained. Data were expressed as mean ± SD. Differences among groups were compared using the Mann–Whitney U-test. Significance was established at $p < 0.05$.

RESULTS

Gene expressions of VEGF and HIF-1α

RT-PCR result showed, mRNA expressions of VEGF and HIF-1α, were not detected in freshly isolated inner or outer meniscal tissues. But in cultured cells, VEGF and HIF-1α mRNA could be detected in both inner and outer meniscal regions. In outer meniscal cells, more VEGF mRNA expression was observed(Fig. 1A). In Real-time PCR, VEGF and HIF-1α gene expression in meniscal tissue and cells were parallel with RT-PCR(Fig. 1B, 1C). Nevertheless, VEGF and HIF-1α mRNA could be detected in both inner and outer cultured injured-meniscal tissue. More VEGF was detected in outer injured meniscal tissue. Meanwhile, HIF-1α was detected in inner and outer injured meniscal tissue similarly(Fig. 2A). Real-time PCR showed a similar result with RT-PCR result(Fig. 2B, 2C).

VEGF deposition in the meniscus

In native meniscal tissue, VEGF was mainly detected in outer and superficial zones by immunohistochemical analyses (Fig. 3A and B). The deposition of VEGF was observed mainly in intracellular, pericellular, and extracellular regions of outer meniscus cells(Fig. 3B). In addition, VEGF was weakly detected in inner

meniscus cells(Fig. 3C). VEGF staining density and VEGF-positive cells count (/2500um^2) were higher in outer region than inner meniscus(Fig. 3E, 3F).

Moreover, in cultured injured meniscal tissue, VEGF was mainly detected around tear region(Fig. 4A, B and C), especially, deposition of VEGF was observed more highly in outer tear region(Fig. 4B) than cultured outer meniscal tissue(Fig. 4D). Moreover, around inner tear area, VEGF deposition arose(Fig. 4C). However, in cultured inner meniscus, VEGF deposition remained low lever(Fig. 4E). These results were parallel with PCR results. VEGF staining density and VEGF-positive cells count(/2500um^2) were higher in the injured region than in the cultured meniscus(Fig. 4G, 4H).

DISCUSSION：

Hypoxia is an established feature in injured tissue and a key stimulus that induces transcription of VEGF[18]. Hypoxia inducibility is conferred on VEGF gene by homologous sequences. A 28-base sequence has been identified in the 5′-promoter of rat and human VEGF gene, which mediates hypoxia-induced transcription[19, 20]. Hypoxia-inducible factor-1α(HIF-1α) is a key mediator of hypoxic responses. In hypoxia condition, HIF1-α accumulates and binds to HIF1-β, thus forming active HIF1 complex that can initiate transcription[21].

In response to hypoxia, accumulation of HIF-1α binds to specific enhancer elements, then recruits additional transcriptional factors such as P-CREB and P-STAT3, resulting in initiating VEGF gene transcription[21]. Hypoxia induces binding of 5′ to the Hypoxia responsive element in VEGF gene promoter region, which in turn increases VEGF transcription[22]. HIF-1α increased VEGF reporter expression in cells exposed to 1 or 20% O_2 in a dose-dependent manner[23]. Lacking HIF-1α, VEGF mRNA levels increased only slightly in response to hypoxia but not induced by hypoxia in cells[23].

Besides the induction of transcription an post-transcription, hypoxia promotes stabilization of the VEGF mRNA by proteins that bind to sequences located in the 3′-untranslated region of the VEGF mRNA[24]. Hypoxia can promote the half-life of VEGF mRNA from 43±6 min under normoxic condition to 106±9 min[24].

In this study, we detected the expressions of VEGF and HIF-1α mRNA in both inner and outer meniscus cells. This result is in accordance with the distribution of perimeniscal capillary plexus. It revealed that VEGF is concerned with meniscus vascularization. And in injured meniscus tissue, expressions of VEGF and HIF-1α mRNA arose, which were not found in native meniscus tissue. We consider the phenomenon is concerned with vascularization of injured meniscus and then promotes meniscal injury healing.

VEGF exists at both inner and outer meniscus cells, but only outer region is vascular area. Angiogenesis is dependent on a balance between pro- and anti-angiogenic factors[25]. VEGF expression in the adult is cell-type-specific[26] and is controlled from transcription to translation, and is upregulated in tumors and various pathologic states[27]. In vitro studies, VEGF can stimulate chondrocytes to proliferate and express MMP-13 via HIF1-α induction, MMP expression might be another factor which inhibits healing despite increased angiogenesis[28].

In our study, we demonstrated that the expression of VEGF and HIF-1α was parallel. According that, we consider that VEGF is also stimulated by HIF-1α. However, HIF-1α and VEGF exist in the inner meniscus, it is still avascular tissue. The anti-angiogenic factors exist in human meniscus, and they may contribute to avascularization of inner meniscus. Fujii et al demonstrated that inner meniscus contained larger amounts of chondromodulin-I(ChM-I) than outer meniscus, and inner meniscus-derived ChM-I inhibited endothelial cell proliferation[29]. In addition, the concentration of endostatin/collagen XVIII which is been demonstrated as an inhibitor of angiogenesis was higher in inner meniscus than outer meniscus[28]. Endostatin/collagen XVIII can inactivate VEGF signal transduction pathways by MAPK Erk 1/2. This anti-angiogenic factor maintained avascular zones in cartilage and fibrocartilage[28]. It may express our contradiction between VEGF distribution

and its angiogenic effect on both inner and outer menisci. Future research are needed for relationship between VEGF and anti-angiogenic factors.

Neovascularization induced by VEGF must be a high level[9], Phillips et al found VEGF implants(200ng to 1000ng) consistently stimulated angiogenesis of rabbit cornea. We consider that in human meniscus, higher VEGF concentration is sufficient to cause the vascularization. Our current study demonstrated that outer meniscus cells expressed higher VEGF detection, this phenomenon may also explain why outer meniscus is vascular but inner meniscus is not. The exact concentration of VEGF between inner and outer meniscus should be measured and compared.

In our meniscal healing model immunostaining, cells counting for VEGF arose approximately 4 folds around injured area compared to uninjured area. We consider those cells are endothelial cells which can induce neovascularization to heal meniscus injury. Previous study found that not all of fibrochondrocytes were positively immunostained in meniscus, fibroblasts and myofibroblasts which are only encountered in injured menisci near the damaged site. These cells may not express VEGF[30].

Limitations of our study are that we were not able to collect tissues from young or healthy individuals. The injury created by a microsurgical blade is not a real tear model. To limit chondrocyte dedifferentiation, we cultured chondrocytes over no more than 3 passages.

In conclusion, our study demonstrates that meniscus contains VEGF in both inner and outer regions. VEGF deposition was highly detected in injured meniscus. Our results suggest that VEGF may be a key angiogenic factor for promoting the healing of meniscal injury, especially in the outer meniscus.

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Figure captions：

Figure 1. Expression of VEGF and HIF-1α mRNA in the superficial zone-excluded meniscal tissues and cultured meniscus cells. RT-PCR result(A) and Real-time PCR result (B, C). *$p < 0.05$.

Figure 2. Expression of VEGF and HIF-1α mRNA in the native, cultured and injured meniscal tissues. RT-PCR result(A) and Real-time PCR result(B, C). *$p < 0.05$.

Figure 3. VEGF localization in native meniscus. Overview of radial section of lateral meniscus(A). VEGF deposition in outer and inner meniscus cells (B, C, brown). Arrows denote VEGF-positive meniscus cells. Control(D). VEGF staining density and VEGF-positive cells count(/2500um2)(E, F). Bars, 100 mm. *$p < 0.05$.

Figure 4. VEGF localization in injured meniscus. Overview of radial section of injured meniscus(A). VEGF deposition around the tear area (B and C, brown). Arrows denote VEGF-positive meniscus cells. VEGF deposition in the inner and outer meniscus cells(D and E, brown). Control (F). VEGF staining density and VEGF-positive cells count (/2500um2)(G, H). Bars, 100 mm. *$p < 0.05$.