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High glucose induces vascular endothelial growth factor production in human synovial fibroblasts through reactive oxygen species generation

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ABSTRACT

Background: Diabetes is an independent risk factor of osteoarthritis (OA). Angiogenesis is essential for the progression of OA. Here, we investigated the intracellular signaling pathways involved in high glucose (HG)-induced vascular endothelial growth factor (VEGF) expression in human synovial fibroblast cells.
Methods: HG-mediated VEGF expression was assessed with qPCR and ELISA. The mechanisms of action of HG in different signaling pathways were studied using Western blotting. Knockdown of proteins was achieved by transfection with siRNA. Chromatin immunoprecipitation assays were used to study *in vivo* binding of c-Jun to the VEGF promoter.
Results: Stimulation of OA synovial fibroblasts (OASF) with HG induced concentration- and time-dependent increases in VEGF expression. Treatment of OASF with HG increased reactive oxygen species (ROS) generation. Pretreatment with NADPH oxidase inhibitor (APO or DPI), ROS scavenger (NAC), PI3K inhibitor (Ly294002 or wortmannin), Akt inhibitor, or AP-1 inhibitor (curcumin or tanshinone IIA) blocked the HG-induced VEGF production. HG also increased PI3K and Akt activation. Treatment of OASF with HG increased the accumulation of phosphorylated c-Jun in the nucleus, AP-1-luciferase activity, and c-Jun binding to the AP-1 element on the VEGF promoter.
Conclusions: Our results suggest that the HG increases VEGF expression in human synovial fibroblasts via the ROS, PI3K, Akt, c-Jun and AP-1 signaling pathway.
General significance: We link high glucose on VEGF expression in osteoarthritis.

1. Introduction

Osteoarthritis (OA) is the leading cause of musculoskeletal handicap in the world [1]. Ageing and obesity are the two main risk factors for OA [2]. However, several epidemiological and experimental data support the hypothesis that diabetes could be an independent risk factor for OA, at least in some patients, leading to the concept of a diabetes-induced OA phenotype [3,4].

OA is a chronic joint disorder characterized by slow progressive degeneration of articular cartilage, subchondral bone alteration, and variable secondary synovial inflammation. In response to macrophage-derived proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), OA synovial fibroblasts (OASF; is most abundant cells in OA joint) produce chemokines that promote inflammation, cartilage degradation, and neovascularization via activation of angiogenesis factors such as vascular endothelial growth factor (VEGF) [5,6]. VEGF is a heparin binding, dimeric glycoprotein that induces the proliferation and migration of endothelial cells to form new vessels, and increases the penetration and extravagation of plasma macromolecules [7,8]. VEGF has shown to play an important role in wound healing, embryonic development, growth of certain solid tumors, and ascites formation [9]. On the other hand, VEGF has been reported to induce in chondrocytes by mechanical overload (the causative factor for OA) [10]. Recently several reports also demonstrated that VEGF was also implicated in the pathogenesis of OA [11,12]. In addition, VEGF isoforms and their receptors (VEGFRs) are expressed in OA cartilage [13,14], and subsequently promoted matrix metalloproteinases expression, leading to cartilage destruction [15]. Treatment with a soluble form of the Flt-1 (VEGF receptor 1) significantly attenuated disease

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severity in arthritis [9,16]. Therefore, anti-angiogenesis may be a novel therapy for OA treatment.

The generation of reactive oxygen species (ROS) plays an important role in diverse cellular functions including signal transduction, oxygen sensing, and high glucose (HG) [17–19]. Among the ROS generating enzymes, NADPH oxidases are the major source of ROS [20]. NADPH oxidase is a multicomponent protein formed by membrane-bound cytochrome b_{558} and composed of the catalytic subunits $gp91^{phox}$ and $p22^{phox}$ and cytosolic regulatory subunits comprised of $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small GTPase Rac [21,22]. ROS production is linked to VEGF expression in endothelial cells and in smooth muscle cells [23,24]. However, the mechanism of HG-induced ROS formation leading to VEGF production in synovial fibroblasts is, so far, unknown.

Angiogenesis is essential for the development, growth, and progression of OA [11]. VEGF is a potent angiogenic factor that is pivotal in the OA pathogenesis. Hyperglycemia is a well recognized pathogenic factor of long-term complications in diabetes mellitus. Although a role of HG in VEGF induction has been implicated in some cell types, the signaling pathway for HG in VEGF production in synovial fibroblasts has not been extensively studied. In this study, we explored the intracellular signaling pathway involved in HG-induced VEGF production in human synovial fibroblasts. The results show that HG activates ROS, phosphoinositide 3-kinase (PI3K), Akt, and AP-1 pathways, leading to up-regulation of VEGF expression.

2. Materials and methods

2.1. Materials

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, PCNA, p-p85, p85, p-Akt, Akt, $p47^{phox}$, p-c-Jun, c-Jun, and the small interfering RNAs (siRNAs) against $p47^{phox}$, c-Jun, and a control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that does not lead to specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pan-Akt inhibitor(1L-6-hydroxymethyl-chiro-inositol-2-((*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) was purchased from Calbiochem (San Diego, CA, USA). Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA). The VEGF enzyme immunoassay kit was purchased from R&D Systems (Minneapolis, MN, USA). The AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). The p85α and Akt1 (Akt K179A) dominant negative mutant were gifts from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, WI). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell cultures

Upon approval by the Institutional Review Board of China Medical University Hospital, and subjects gave informed written consent. Human synovial fibroblasts were isolated using collagenase treatment of synovial tissues obtained from knee replacement surgeries of 35 patients with OA. Fresh synovial tissues were minced and digested in a solution of collagenase and DNase. Isolated fibroblasts were filtered through 70-μm nylon filters. The cells were grown on plastic cell culture dishes in 95% air/5% CO_2 in RPMI 1640 (Life Technologies) that was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (pH adjusted to 7.6). Fibroblasts from passages four to nine were used for the experiments [25–27].

2.3. Measurement of VEGF production

Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluency, cells were treated with HG (33 mM) and then incubated in a humidified incubator at 37 °C for 24 h. To examine the downstream signaling pathways involved in HG treatment, cells were pretreated with various inhibitors for 30 min (These inhibitors did not affect cell viability; Supplemental data Fig. S1) before addition of HG (33 mM) administration. After incubation, the medium was removed and stored at −80 °C until the assay was performed. VEGF in the medium was assayed using VEGF enzyme immunoassay kits, according to the procedure described by the manufacturer.

2.4. Quantitative real-time PCR

Total RNA was extracted from synovial fibroblasts with a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA (in 2 μl RNase-free water) that was reverse transcribed into cDNA with an MMLV RT kit (Promega, Madison, WI) and following the manufacturer's recommended procedures [28,29]. The reverse transcription reaction mixture was incubated at 37 °C for 60 min and then at 70 °C for 5 min to inactivate MMLV. Quantitative real time PCR (qPCR) analysis was carried out with TaqMan® one-step PCR Master Mix (Applied Biosystems, Foster City, CA). cDNA template (2 μl) was added to each 25-μl reaction with sequence-specific primers and TaqMan® probes. All target gene primers and probes were purchased commercially (β-actin was used as internal control) (Applied Biosystems). qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were: 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

2.5. Western blot analysis

Cellular lysates were prepared as described [30,31]. Proteins were resolved using SDS-PAGE and transferred to Immobilon polyvinyldifluoride membranes. The membranes were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit antibodies against human p-p85, p85, p-Akt, Akt, p-c-Jun, or c-Jun (1:1000) for 1 h at room temperature. After three washes, the blots were incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). The activities of Akt were determined using kit from Cell Signaling Technology according to the manufacturer's instructions.

2.6. ROS generation assay

The fluorescent probe DCF-DA was used to monitor net intracellular accumulation of ROS. This method is based on the oxidative conversion of nonfluorescent DCFH-DA to fluorescent DCF by H_2O_2. OASF cells were washed with warm Hank's Balanced Salt Solution (HBSS) and incubated in HBSS or cell medium containing 10 mM DCFH-DA at 37 °C for 45 min. Subsequently, HBSS or cell medium containing DCFH-DA was removed and replaced with fresh cell medium. OASF cells were then incubated with HG. The fluorescence intensity (relative fluorescence units) was measured at 485 nm excitation and 530 nm emission using a fluorescence microplate reader (Appliskan™, Thermo, Fremont, CA, USA).

2.7. NADPH oxidase activity

After incubation with HG for indicated time intervals, cells were gently scraped and centrifuged at 400 g for 10 min at 4 °C. The cell pellet was resuspended with RPMI-1640 medium, and the cell suspension was kept on ice. To a final 200 μL volume of RPMI-1640 medium containing either NADPH (1 μM) or lucigenin (20 μM), 5 μL of cell suspension (0.2×10^5 cells) were added to initiate the reaction followed