Inhibition of histone deacetylase down-regulates the expression of hypoxia-induced vascular endothelial growth factor by rheumatoid synovial fibroblasts

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Abstract. Objective: To investigate the effect of FK228 on the in vitro expression of hypoxia-inducible factor-1 alpha (HIF-1α) and vascular endothelial growth factor (VEGF) by rheumatoid arthritis synovial fibroblasts (RASFs), and on the in vivo expression of VEGF and angiogenesis in the synovial tissue of mice with collagen-antibody-induced arthritis (CAIA).

Methods: RASFs were stimulated with IL-1β and TNFα and then incubated under hypoxia (1 % O2) with various concentrations of FK228. The effects of FK228 on the expression of HIF-1α and VEGF mRNA were examined by quantitative real-time PCR. Changes in HIF-1α protein expression and the secretion of VEGF protein into the culture medium were examined by Western blot analysis and ELISA, respectively. Immunohistochemical analysis was carried out to investigate the expression and distribution of VEGF in synovial tissues of CAIA mice.

Results: The cytokine-stimulated expression of HIF-1α and VEGF mRNA was inhibited by FK228 in a dose-dependent manner. FK228 also reduced the expression of HIF-1α and VEGF protein. Intravenous administration of FK228 (2.5 mg/kg) suppressed VEGF expression, and also blocked VEGF protein expression and angiogenesis in the synovial tissue of CAIA.

Conclusion: FK228 may exhibit a therapeutic effect on RA by inhibition of angiogenesis through down-regulation of angiogenesis related factors, HIF-1α and VEGF.

Key words: Histone deacetylase (HDAC) – Rheumatoid arthritis – Vascular endothelial growth factor (VEGF) – Hypoxia-inducible factor-1 (HIF-1)

Introduction

Angiogenesis is an essential component in the formation and maintenance of inflammatory synovial tissues in rheumatoid arthritis (RA) [1] as it allows these tissues to cope with the increased demand for oxygen and nutrients [2]. Previous studies demonstrated that the inhibition of angiogenesis ameliorated synovial inflammation in animal models of arthritis [3], suggesting that blockade of angiogenesis offered a promising therapeutic strategy for RA. However, the patho-mechanisms that control the development of synovial angiogenesis in RA are not fully understood.

Several growth factors, including fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), as well as soluble forms of several adhesion molecules are able to stimulate angiogenesis directly by interacting with endothelial cell receptors [4]. VEGF is of particular importance in the process of angiogenesis as it promotes endothelial cell migration and increases vascular permeability [5]. It is known that RA synoviocytes secrete VEGF, and synovial fluids in RA patients contain abnormally high levels of VEGF [6, 7]. Accordingly, the VEGF receptors flt-1 and fkl-1 are strongly expressed in endothelial cells of the RA synovium [4]. In clinical studies, administration of anti-TNFα antibody reduced serum levels of VEGF by up to 40 % in patients with RA; however, circulating VEGF levels remained significantly higher than in healthy individuals [2]. Therefore, anti-angiogenic agents, including antibodies to VEGF and VEGF receptor antagonists, are currently being tested for their therapeutic use in RA [8–12].

There are many angiogenic and angiostatic factors that regulate VEGF expression [5]. In all cell types studied to date, two transcription factors, hypoxia-inducible factor-1 (HIF-1) and HIF-2, which are induced to similar levels...
under hypoxic conditions, were shown to stimulate the VEGF gene promoter. While the HIF-1α subunit is rapidly degraded under normoxic conditions, under hypoxic conditions, it is stabilized and translocates to the nucleus, where it transactivates a number of genes with hypoxia-responsive elements in their promoters [13]. The HIF-2α subunit is highly expressed by vascular endothelial cells and activates the transcription of endothelial-cell-specific receptor tyrosine kinase and the VEGF receptor flk-1. A previous study demonstrated significant cytoplasmic and nuclear overexpression of HIF-1α and HIF-2α in the synovial lining and stromal cells in RA and in osteoarthritis synovial tissue [14]. More recently, Makino et al. reported that CD3-positive T cells which had accumulated in inflammatory tissue expressed HIF-1α [15]. The authors postulated that hypoxia plays an important role in the survival of activated T cells via the HIF-1α-adrenomedullin cascade. These findings suggest that HIF-1α is closely involved in synovial pathology and can thus serve as a therapeutic target for RA.

It has been shown that histone modification through reversible acetylation is a crucial event in gene expression [16]. Histone acetylation is controlled by two enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC) [17, 18]. Increasing evidence indicates that the antitumor activity of HDAC inhibitors is exerted through multiple mechanisms, such as apoptosis, cell cycle arrest, and differentiation, via the modulation of gene expression [19–24]. Recent reports demonstrated that specific HDAC inhibitors, including trichostatin A (TSA) and depsipeptide (FK228), inhibit angiogenesis by altering HIF-1 expression and VEGF signaling [25–27]. This finding raised the question whether HDAC inhibitor prevents angiogenesis within the inflammatory joint by repressing hypoxia-induced HIF-1 and VEGF expression by rheumatoid arthritis synovial fibroblasts (RASFs). To answer that question, we investigated the in vitro effects of FK228, a specific HDAC inhibitor, on the expression of HIF-1α and VEGF by RASFs under hypoxic conditions. In addition, the in vivo effects of FK228 on the expression of VEGF and the number of blood vessels in synovial tissue were studied in mice with collagen-antibody-induced arthritis (CAIA). The results demonstrated a potential for beneficial role of HDAC inhibitors in blockage of angiogenesis via suppression of angiogenesis-related factors in RA synovial tissue.

**Materials and methods**

**Reagents**

Recombinant human IL-1β and TNFα were purchased from R&D Systems (Minneapolis, MN), stored at −80°C, and diluted in culture medium immediately before use. Mouse monoclonal antibody against HIF-1α was purchased from Novus Biologicals, Inc. (Littleton, CO). Rabbit polyclonal antibody against VEGF (A-20) was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). FK228 was provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). For the in vitro studies, FK228 was dissolved in DMSO and diluted with each of the experimental media before use. For the in vivo studies, FK228 was dissolved in and diluted with 10% polyoxyethylene (60)-hydrogenated castor oil in saline (HCO60 saline).

**Isolation and culture of human RASFs**

Following the written permission of the patients, fresh synovial tissues were obtained from five RA patients during total joint replacement surgery. The tissues were minced and then immediately digested with collagenase (Wako, Osaka, Japan) and DNAase (Sigma-Aldrich) at 37°C, as previously described [28]. Tissue debris was removed with a cell strainer, and the remaining cells were washed twice with Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% HEPES (Life Technologies, Tokyo, Japan), 100U penicillin/ml, and 100μg streptomycin/ml (Life Technologies). The resultant single-cells were dispensed into the wells of a 24-well microtiter plate (Costar, Cambridge, MA) at a density of 2×10^6 cells/ml in 2ml of DMEM supplemented with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD), 100U penicillin/ml, and 100μg streptomycin/ml. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Synovial-tissue cell cultures were divided once weekly until the primary cultures had reached confluence. After the third passage, morphologically homogeneous fibroblast-like cells were obtained.

**Hypoxic conditions**

A sealed chamber (ASTEC APM-30D, Fukuoka, Japan) was used to culture cell preparations in a low-oxygen-tension environment of 1% O₂, 5% CO₂, and 94% N₂.

**Quantitative real-time PCR for the detection of HIF-1α and VEGF mRNA**

Cells were seeded at a density of 1×10⁶/well in 6-well culture dishes, stimulated with recombinant human IL-1β (1 ng/ml) and recombinant human TNFα (10 ng/ml) for 1 h, and then incubated with or without various concentrations of FK228 under 1% O₂ for up to 24 h. The morphology of the cells was examined under polarized light microscopy, after which total RNA was isolated from cultured cells using Isogen reagent (Nippon Genhe, Toyama, Japan). The purified RNA was reverse-transcribed using Rever Tra Ace (Toyobo, Tokyo, Japan).

For real-time PCR, the primer sequences of HIF-1α and VEGF were as follows: for HIF-1α, 5′-ATC ATG CAG CTA CTA CAT CA-3′ (forward) and 5′-CTT CAC AAT CAT AAC TGG TC-3′ (reverse); for VEGF, 5′-TCT TCA AGC CAT CCT GTG T-3′ (forward) and 5′-CTT TCT TTG GTG TGC ATT C-3′ (reverse); for β-actin, 5′-TTC GGC ATG GAG TCC T-3′ (forward) and 5′-AGG AGC AAT GAT CTT GAT C-3′ (reverse). Real-time PCR reactions were carried out using a LightCycler FastStart DNA Master SYBR green I kit (Roche Molecular Biochemicals, Mannheim, Germany) as recommended by the manufacturer. Gene expression was quantified by dividing the level of HIF-1α and VEGF mRNA expression by the level of β-actin mRNA expression.

**Analysis of HIF-1α and VEGF protein expression**

Cells were seeded at a density of 2×10⁶ cells/well in 6-well culture dishes, stimulated with TNFα and IL-1β as described above, and then incubated with FK228 (5nM) under hypoxia for up to 24 h (0, 12, and 24 h). For analysis of HIF-1α, the cells were washed twice with PBS, scraped, and lysed, after which proteins were extracted in a buffer of ice-cold 1% Triton X-100 in PBS supplemented with 1mM PMSF, 2mM N-ethylmaleimide, 5mM iodoacetamide, and 1mM EDTA. The resulting extract was incubated on ice for 5 min and centrifuged at 15,000 g for 10 min at 4°C. The concentrations of proteins in the supernatant were measured and equalized using a Bio-Rad protein assay kit. Forty μg of protein per lane were run on 8% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 1% bovine serum albumin (BSA) in PBS, incubated with primary antibody diluted in blocking so-