Secretion of Vascular Endothelial Growth Factor is Inhibited by the Antioxidant of N-acetylcysteine in Fibroblast-like Synoviocytes from Rheumatoid Arthritis

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ABSTRACT

The aim of this study was to investigate the effects of the anti-oxidant N-acetylcysteine (NAC) on the levels of vascular endothelial growth factor (VEGF) in human fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA). RA-FLS were obtained from three RA patients who were undergoing knee joint replacement. Passaged RA-FLS were treated with or without NAC (1 mM) for 24 h. VEGF protein was secreted into the conditioned medium and the expression levels of VEGFA mRNA and VEGF protein by the RA-FLS also analyzed. Secreted VEGF was significantly reduced in the conditioned media of RA-FLS after NAC treatment in two different culture media (10% FBS; p = 0.0028 and 0.2% FBS; p = 0.0003). NAC did not reduce the expression levels of VEGFA mRNA and VEGF protein in the RA-FLS was not reduced remarkably, suggesting that NAC inhibited VEGF protein secretion by the RA-FLS. NAC is a potent inhibitor of VEGF protein secretion by RA-FLS. This finding indicated that this compound is a candidate therapeutic agent for treating RA synovitis in the future.

Key words: Rheumatoid arthritis, vascular endothelial growth factor, N-acetylcysteine, fibroblast-like synoviocytes.

INTRODUCTION

Angiogenesis in the synovium during the course of rheumatoid arthritis (RA) begins at an early stage of the disease and is essential for progression of the synovitis (Koch, 1998). Synovial hyperplasia as well as, infiltration of inflammatory cells in affected joints in RA is accompanied by increased tissue formation and subsequent local hypoxia.

Moreover, it has been recently reported that the hypoxic condition is a key regulator of both angiogenesis and inflammation in RA (Konisti et al., 2012), which is thus likely to cause a vicious cycle that results in chronic and persistent inflammation. Among hypoxia-inducible molecules, vascular endothelial growth factor (VEGF), one of the best-characterized hypoxia response element (HRE) induced genes, is a key regulator of angiogenesis (Ferrara et al., 2003), cell migration (Duchek et al., 2001) and inflammation (Cursiefen et al., 2004). Activation of hypoxia-inducible factor (HIF), a transcription factor that is expressed in response to hypoxia, significantly contributes to the induction of VEGF for angiogenesis and increased levels of VEGF in serum, synovial fluid and tissue in RA have been reported (Koch et al., 1994; Fava et al., 1994; Ballara et al., 2001; Kurosaka et al., 2010; Nakahara et al., 2003). Synovial hypoxia, therefore, likely contributes to RA by promoting inflammation, angiogenesis and cellular infiltration.

The antioxidant N-acetylcysteine (NAC) was recently identified as a novel inhibitor of the hypoxic response pathway (Gao et al., 2007). NAC is a precursor of glutathione, a major anti-oxidant in the body. NAC has
been tried as an anti-tumorigenic agent due to its ability to remove reactive oxygen species (ROS), which induce DNA damage and genomic instability (Albini et al., 2001). ROS are produced under hypoxic stress by cells and are capable of inactivating the prolyl hydroxylases (PHDs) essential for the catalytic hydroxylation of proteins. Thus, ROS prevent the hydroxylation of HIF-1α. Reducing ROS through administration of NAC was shown to restore PHDs activity and prevent HIF-1α stabilization under hypoxia (Gao et al., 2007; Calvani et al., 2012).

Taking these emerging data together, we hypothesized that NAC may play an effective role in anti-inflammation in RA by inhibiting the hypoxic response pathway. However, no study has been performed on the relationship between NAC and inflammation in RA. In the present study, we found that NAC was a down regulator of VEGF secretion in RA-FLS.

MATERIALS AND METHODS

Preparations of synovial tissue and cell culture

Synovial tissues were obtained from three patients undergoing knee joint replacement due to joint destruction. These patients had been diagnosed according to the revised criteria of the American College of Rheumatology (Arnett et al., 1988). Written informed consent was obtained from each patient before the specimens were taken in accordance with the local ethics committee (Niigata University Medical and Dental Hospital). FLS isolation was performed principally according to the report by Rosengren et al. (2007). Briefly, synovial tissues were cut into small pieces and digested with type 1 collagenase (3 mg/ml) in MEM-α (Gibco, Life Technology) for 3 h. The tissue was then filtered using a 70 μm nylon cell strainer, washed, and suspended in MEM-α. Dissociated cells were then centrifuged at 3000 rpm for 3 min, re-suspended in MEM-α supplemented with 10% fetal bovine serum (FBS) containing 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA, USA). After an overnight culture, the non-adherent cells were removed, and the adherent cells cultivated in MEM-α supplemented with 10% FBS containing 1% antibiotic/antimycotic solution (culture medium). RA-FLS from passages four to eight were used in each experiment confirming that the cells were morphologically homogenous and exhibited the appearance of FLS with a typical bipolar configuration under inverse microscopy. After culture for 4 h to allow cell attachment, the cells were incubated with or without N-acetylcysteine (NAC; Sigma-Aldrich, St. Louis, MO, USA) for 24 h in culture medium containing either 10% FBS (culture medium) or 0.2% FBS (starvation medium). The low serum-containing medium was used to minimize the influence of serum factors. Since we already knew that 1 mM NAC did not reduce RA-FLS cell viability in both media (data not shown), we adopted this concentration for the NAC-treated group.

Enzyme-linked immunosorbent assay (ELISA)

The supernatants of treated RA-FLS were collected. VEGF protein was measured by the sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems) according to the manufacturer’s protocol.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cultured cells using the ISOGEN Reagent (Nippon Gene, Toyama, Japan), and cDNA was generated using the Prime Script RT reagent kit (Takara Bio, Tokyo, Japan), according to the manufacturer’s protocol. Quantitative real-time PCR was carried out using a Thermal Cycler Dice Real Time System (Takara Bio) and SYBR Premix Ex Taq II (Perfect Real time) PCR kit (Takara Bio) according to the manufacturer’s protocol. The primer sequences used were as follows: VEGFA, 5′-TCACAGTACAGGATGAGGACAC-3′ (forward) and 5′-CAAAGCACACAGCTGTTGAAG-3′ (reverse); GAPDH, 5′-GCCCTGACGTGAAG-3′ (forward) and 5′-TGGTAAGACGCAAGG-3′ (reverse). DNA amplification consisted of denaturation at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing and extension at 60°C for 30 s. Non-treated cells were used as positive control for VEGFA gene expression. Data were analyzed using the Thermal Cycler Dice Real Time System analysis software package (Takara Bio). After the PCR results were normalized to those of GAPDH, the relative mRNA expression level of each sample was calculated.

Western blotting

Treated RA-FLS were washed twice with cold PBS and harvested with a cell scraper. For preparation of whole cell lysates, cell pellets were extracted with lysis buffer (62.5 mM Tris (pH 6.8), containing 2% SDS, 5% glycerol, 6 M urea), and 4 μl protease inhibitor cocktail (Complete; Roche Diagnostic, Mannheim, Germany). After measurement of the supernatant protein concentration using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), the supernatants were mixed with 5% (v/v) 1 M DTT and 5% (v/v) bromophenol blue and heated at 95 to 100°C for 5 min. Equal amounts (50 μg/lane) of proteins were separated by SDS-PAGE 10% gels and then electrotransferred to nitrocellulose membranes. The membranes were incubated with the primary antibodies before being probed with HRP-linked secondary antibodies (Amersham Biosciences, Little Chalfont, UK) and developed using ECL detection reagents (American Biosciences). The following antibodies were used: rabbit anti-VEGF (A-20) polyclonal antibodies (1: 1000; Santa Cruz Biotechnology, Santa Cruz,
CA, USA) and mouse anti-β actin mAb (1: 3000; Sigma-Aldrich). The levels were quantified using Image J, and normalized to that of β actin. Then, the relative protein levels of VEGF were calculated.

Statistical analysis

Wilcoxon t-test was used to calculate p values for paired samples. For unpaired samples, Mann-Whitney U-test was used. GraphPad PRISM software (GraphPad, La Jolla, CA) was used to calculate the p values. Data represented means ± SE. A p value of < 0.05 was considered to indicate statistical significance.

RESULTS

NAC reduced secreted VEGF protein levels dramatically in the conditioned media

To investigate the possible inhibitory effects of NAC (1 mM) on VEGF production by RA-FLS, RA-FLS were seeded at 1 to $3 \times 10^5$ cells/ml in each medium. Thereafter, the secreted VEGF protein in the media from the RA-FLS treated with either NAC (1 mM) or not (Cont) was measured using an ELISA (7 replicates in A, 6 replicates in B), respectively. The average concentration of VEGF protein in the media was dramatically down-regulated by NAC (1 mM) treatment compared with that in the control. (A) Culture medium; (B) starvation medium. The Wilcoxon t-test was used in the statistical analysis.

Figure 1. Secreted VEGF protein in the media from RA-FLS treated with either NAC (1 mM) or not (Cont) was measured using an ELISA (7 replicates per data point in the culture medium, 6 replicates per data point in the starvation medium), respectively. Figure 1 shows that in both media groups, the average concentration of VEGF protein in the media was dramatically down-regulated by NAC treatment as compared with that in the control (925.9 pg/ml in the control group, and 518.1 pg/ml in the NAC-treated group; p = 0.0028 in the culture medium, 221.7 pg/ml in the control group, and 77.3 pg/ml in the NAC-treated group; p = 0.0003 in the starvation medium).

NAC treatment did not affect the expression levels of VEGFA mRNA by RA-FLS

VEGF isoform A (VEGFA) is the most abundant variant of the VEGF family. To investigate whether NAC (1 mM) also had effects on VEGFA mRNA expression, the expression levels of VEGFA mRNA in RA-FLS treated with or without NAC (1 mM) for 24 h were analyzed. RA-FLS were seeded at $1 \times 10^5$ cells/ml for 4 h to allow attachment followed by treatment with either NAC (1 mM) or not (Cont) for 24 h in both culture medium and starvation medium. Thereafter, cell lysates were collected and subjected to qPCR analysis.
The qPCR was performed thrice with quadruplicate measurements for each condition. As shown in Figure 2, the expression levels of VEGFA mRNA were not decreased in RA-FLS treated with NAC (1 mM) in either medium.

**NAC treatment did not reduce the production of VEGF protein by RA-FLS**

The production of VEGF protein by RA-FLS was analyzed by western blotting. As earlier described, RA-FLS were seeded at $1 \times 10^5$ cells/ml and incubated for 4 h to allow attachment followed by treatment with either NAC (1 mM) or not (Cont) for 24 h in both culture medium and starvation medium. Thereafter, cell lysates were collected and subjected to western blotting analysis. Experiments were performed thrice for each condition. The results showed that the VEGF protein produced by RA-FLS existed in the form of a dimer (42 kDa) and that the VEGF protein levels produced by RA-FLS treated with NAC (1 mM) were slightly decreased in both media (no statistical significance) (Figure 3).

**DISCUSSION**

The levels of secreted VEGF protein were significantly inhibited in the conditioned medium of RA-FLS after NAC (1 mM) treatment as detected by ELISA (Figure 1). However, NAC did not reduce the cellular expression levels of VEGFA mRNA in RA-FLS, and VEGF protein levels in RA-FLS were not decreased significantly either. These findings suggest that NAC played a key role in inhibiting the secretion of VEGF by RA-FLS into the conditioned medium. In accordance with our results, Sceneay et al. (2013) reported that NAC treatment was able to reduce the secretion of VEGF protein in response to hypoxia in breast tumor cells in vitro, whereas the gene expression of VEGF was not affected. Although, the regulation of VEGF expression at the transcriptional, post-transcriptional and translational levels has been extensively studied (Akiri et al., 1998; Huez et al., 1998; Chung et al., 2002; Mezquita et al., 2005), our findings focused on the regulation of secretion by RA-FLS.

It has been reported that VEGF$^{165}$, the most predominant VEGF isoform, has a direct pro-inflammatory role in the pathogenesis of RA (Yoo et al., 2005) and that the VEGF$^{165}$ receptor is expressed in RA synovium (Yoo et al., 2009; Ikeda et al., 2000). Recently, Guzman-Hernandez et al. (2014) reported that VEGF$^{165}$ is secreted in part through shedding from the cell surface together with membrane components. Proteolytic processing in both the shedding and release of membrane proteins can function as a post-translational switch that regulates the activity of the cleaved substrate. This process might activate or inactivate the substrate protein or substantially change its functional
properties. Disintegrin and metalloprotease (ADAM) proteins are key components in this shedding process (Blobel, 2005).

Indeed, the expression of ADAM10, ADAM15, and ADAM17 in arthritic cartilage and synovial tissues has been demonstrated (Pruessmeyer and Ludwig, 2009; Bohm et al., 2001; Komiya et al., 2005). Intriguingly, it is reported that the expression of both ADAM10 and ADAM17 is increased in response to hypoxia (Rzymski et al., 2012; Barsoum et al., 2011).

As earlier described, NAC was recently identified as a novel inhibitor of the hypoxic response pathway (Gao et al., 2007). Taking these findings together, we believe that NAC inhibits the hypoxic response pathway, consequently decreases the expression levels of ADAMs that play key roles in ectodomain protein shedding and inhibits the

Figure 3. (A and B) VEGF protein in RA-FLS treated with either NAC (1 mM) or not (Cont) for 24 h was analyzed by western blotting. The representative figure shows that VEGF protein in RA-FLS existed in the form of a dimer (42 kDa). (C and D) Relative expression levels of VEGF protein in RA-FLS were compared with the controls (Cont) that were standardized to value of 1.0. (A and C) culture medium, (B and D) starvation medium. Experiments were performed thrice for each condition. The Mann-Whitney U-test was used in the statistical analysis.
secretion of VEGF protein by RA-FLS. However, more studies on the influence of NAC on VEGF protein in RA-FLS are needed to elucidate its effectiveness in the future.

In summary, we have demonstrated that NAC inhibits secretion of VEGF protein by RA-FLS, suggesting that NAC might have beneficial effects on both preventing and treating RA. Therefore, our results indicated that NAC is a promising candidate inhibitor for RA synovial angiogenesis and inflammation.

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REFERENCES


