Background: Matrix metalloproteinases (MMPs) play an important role in degradation of the extracellular matrix of injured tissue. MMP-9 expression increases in fibrillating atrial tissue; however, the mechanism for this increase has not been clarified.

Methods and Results: Changes in the expression of vascular endothelial growth factor (VEGF), VEGF receptors, and hypoxia-induced transcription factor-1α (HIF-1α) in fibrillating atrial tissue were investigated. Atrial tissue samples were obtained from 13 patients with atrial fibrillation (AF) and 25 patients without a history of AF (regular sinus rhythm, RSR) undergoing cardiac operations. Western blot, real-time polymerase chain reaction, and immunofluorescence analyses of the expression of VEGF, VEGF receptors, and HIF-1α were performed. The VEGF mRNA and protein levels increased significantly in the AF group compared with the RSR group (P<0.05), and the expression of HIF-1α protein was also significantly higher in the AF group. VEGF receptor-1 mRNA, a high-affinity receptor for VEGF, but not VEGF receptor-2 mRNA, was upregulated in the atria of the AF group (P<0.05). Immunofluorescence staining revealed excess production and co-localization of HIF-1α, VEGF and MMP-9 in the endothelium of the atrial arteries in the AF group.

Conclusions: It is possible that upregulation of HIF-1/VEGF is involved in the enhancement of MMP-9 expression under hypoxic conditions. (Circ J 2010; 74: 1815–1821)

Key Words: Atrial fibrillation; Hypoxia; Hypoxia-induced transcription factor-1α (HIF-1α); Matrix metalloproteinase 9 (MMP-9); Vascular endothelial growth factor (VEGF)

Atrial fibrillation (AF) is a common arrhythmia associated with an increased risk of stroke, cardiac failure, and mortality. In almost 80% of patients, AF is associated with organic heart disease, including valvular heart disease (mostly mitral valve disease), coronary artery disease, hypertensive heart disease, hypertrophic and dilated cardiomyopathy, and congenital heart diseases such as atrial septal defects. Electrical remodeling caused by alterations in the transmembrane ionic currents and shortening of the atrial effective refractory period might lead to an increase in the stability of AF within a few hours to a few days of the event. Underlying structural remodeling might occur before, during, and after the electrical remodeling, and play an important role in the progression of sustained AF. There have been several reports on the mechanism and role of structural remodeling in AF. Remodeling of the cellular ultrastructure, such as myolysis in the atrial myocardium, develops progressively during AF. An increase in the expression of gap junctions (connexin 43) has been reported to induce changes in the biophysical properties of the atrial tissue in AF patients. Enhanced metalloproteinase–disintegrins activity has also been reported to contribute to the dilatation of the fibrillating human atria.

Matrix metalloproteinases (MMPs) are a family of zinc metalloendopeptidases that play an important role in the physiological and pathological turnover of matrix components, tissue degradation and repair. Extracellular matrix degradation by MMPs is involved in the pathogenesis of cardiovascular diseases, including atherosclerosis, restenosis, dilated cardiomyopathy, and myocardial infarction. Of the MMPs,

Received December 9, 2009; accepted April 20, 2010; released online July 10, 2010 Time for primary review: 27 days Department of Cardiovascular Medicine, Hiroshima University Graduate School of Biomedical Science, Hiroshima (H.O., Y.N., Y.H., K.S., Y.T., N.O., Y.M., S.U., K.K., Y.K.); Research Institute, National Center for Geriatrics and Gerontology, Nagoya (S.N.); Department of Cardiology, Hiroshima City Asa Hospital, Hiroshima (K.D.); Department of Surgery, Hiroshima University Graduate School of Biomedical Science, Hiroshima (K.I., T.S.); and Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Science, Hiroshima (K.C.), Japan. Mailing address: Yukiko Nakano, MD, Department of Cardiovascular Medicine, Hiroshima University Graduate School of Biomedical Science, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: ynakano@xj8.so-net.ne.jp


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MMP-9 is a major protease and is implicated in myocardial matrix remodeling and could be a target for the prevention or treatment of the failing heart.\textsuperscript{13}\textsuperscript{14}\textsuperscript{17}\textsuperscript{21} We previously demonstrated that MMP-9 expression increases in fibrillating atrial tissue, which may contribute to the atrial structural remodeling of AF.\textsuperscript{21} However, the mechanism of the increase in MMP-9 expression in fibrillating atrial tissue remains unknown.

MMP-9 is known as a modulator of vascular endothelial growth factor (VEGF) induction and angiogenesis.\textsuperscript{23}\textsuperscript{24} Paradoxically, several studies have demonstrated that VEGF stimulates the expression of MMPs, including MMP-9.\textsuperscript{25}\textsuperscript{26} In addition, it has been reported that hypoxic MMP-9 expression in human retinal pigment epithelial cells is mediated by VEGF signaling.\textsuperscript{27} Hypoxia-induced VEGF expression is strongly regulated by hypoxia-inducible factor 1 (HIF-1), the transcriptional factor for VEGF, which is a critical modulator in various organisms for sensing and responding to changes in the oxygen concentration.\textsuperscript{28}\textsuperscript{32} Therefore, we hypothesized that hypoxia-dependent activation of HIF-1 would increase the level of VEGF and, subsequently, induce MMP-9 expression. In this study, we examined the expression levels of VEGF and HIF-1 in fibrillating atrial tissue.

**Methods**

**Tissue Collection**

Right atrial appendages were obtained from 38 patients undergoing cardiac operations, including 25 with regular sinus rhythm (RSR) and no history of AF, and 13 with AF (paroxysmal AF, 6 patients; chronic AF, 7 patients). The Ethics Committee of the Graduate School of Biomedical Science, Hiroshima University approved all procedures involving the use of human tissue. Informed consent was given by the patients before tissue harvesting. A transverse section (5–8 mm) of the atrial appendages was divided into 3 sections, 1 of which was embedded in OCT compound (Sakura, Torrance, CA, USA) and frozen with liquid nitrogen. The tissue blocks were stored at –80°C until sectioning. Another section was quickly frozen with liquid nitrogen and stored at –80°C for later protein extraction. The 3rd section was preserved in RNA later™ (Ambion, Austin, TX, USA) at 4°C and used to extract the RNA.

**Western Blotting Analysis**

Frozen cardiac tissue samples were homogenized in a lysis buffer [20 mmol/L Tris, 1% Triton X, 10% glycerol, 1% DOC, 0.1% SDS, 50 mmol/L NaF, 10 mmol/L NaF-O
\textsubscript{4}, 1 mmol/L DTT, 1 mmol/L banadate, 10 μg/ml leupeptin (pH 7.4)] with a Zilconia bead at 30 femo to second and 4°C for 8 min using MM 300 (Qiagen, Hilden, Germany), and then centrifuged at 14 krpm at 4°C for 10 min. The protein concentrations of the supernatants were determined by a DC protein assay (Bio-Rad, Richmond, CA, USA). Equal amounts of protein (2 μg) were separated by an SDS-PAGE and immunoblotted onto a nitrocellulose membrane with anti-VEGF antibodies (Research Diagnostics Inc, Concord, MA, USA) and anti-HIF-1α antibodies (NOVUS Biologicals, Littleton, CO, USA). The VEGF and HIF-1α protein bands were visualized using horseradish peroxidase-conjugated anti-IgG secondary antibodies and ECL plus western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK). The membranes were washed, dried, and exposed to an X-ray film (Kodak). The images were analyzed using NIH Image freeware (http://rsb.info.nih.gov/nih-image/index.html) and quantified by normalizing them to β-actin.

**Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)**

The tissue samples preserved in the RNA later™ were pulverized in a Trizol® reagent (1 ml/100 mg tissue), and the total RNA was extracted using the Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Precisely, 1 μg of the total RNA from each sample was reverse transcribed to generate cDNA using a Super Script III TM Reverse Transcriptase (Invitrogen). The qRT-PCR was performed using a Light Cycler (Roche, Mannheim, Germany).

We used 1 mol/L of cDNA for the PCR mixture (Light Cycler™ Fast Start DNA Master SYBR Green I kit; Roche), which contained 0.5 μmol/L of the original specific primers for VEGF, HIF1-α, HIF-1 oxygen-dependent degradation domain, and VEGF receptor (VEGFR)-1 and VEGFR-2. An initial denaturation at 95°C for 10 min was followed by amplification, involving 35 cycles with denaturation at 95°C for 10s, annealing at 60°C for 10s, and elongation at 72°C for 16s. All the samples were analyzed in triplicate and quantified by normalizing them to β-actin. The fluorescence intensity of the SYBR Green I reflected the quantity of PCR product actually formed.

**Immunofluorescence Staining for VEGF, HIF-1α and MMP-9**

Frozen sections of the right atrial appendage were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.5% Triton X-100/PBS for 5 min. The sections were blocked with 1% bovine serum albumin (BSA)/PBS and incubated with rabbit anti-VEGF (Chem-icon, Rosemont, IL,
USA)/mouse anti-HIF-1α (NOVOS) and mouse anti-VEGF (RDI)/rabbit anti-MMP-9 (Oncogene Science, Cambridge, MA, USA) antibodies diluted 1:100 concentration in 1% BSA/PBS. Alexa 488 conjugated goat anti-mouse and Alexa 594 conjugated goat anti-rabbit antibodies (Invitrogen) were used as secondary antibodies. All antibody incubations were performed at 37°C for 30 min. The nuclei were stained with 10 μmol/L of Hoechst 33342. The samples were examined using a Leica DMRE epifluorescence microscope or Zeiss Axioplan with an Apotom. Images were taken by a CCD camera and Adobe Photoshop® (Adobe Systems, Inc, San Jose, CA, USA) was used for the presentation of the images.

**Statistical Analysis**

The quantitative data from the AF and RSR groups were compared by Mann-Whitney U test. The Kruskal-Wallis test was used to compare the data from the 3 groups (RSR, paroxysmal AF, and chronic AF). The data are presented as the mean±SD. A P-value <0.05 was considered statistically significant.

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**Figure 1.** Relative expression of the VEGF and HIF-1α mRNA. The relative expression of VEGF mRNA was higher in the AF group than in the RSR group; however, the mRNA expression levels of HIF-1α and the HIF-1 ODD domain (data not shown) were similar in the 2 groups. AF, atrial fibrillation; HIF-1α, hypoxia induced transcription factor-1α; ODD, oxygen-dependent degradation; RSR, regular sinus rhythm; VEGF, vascular endothelial growth factor.

**Figure 2.** Relative expression of VEGFR-1 and VEGFR-2 mRNA. The relative mRNA expression of VEGFR-1, but not VEGFR-2, was higher in the AF group than in the RSR group. *P<0.05, AF vs RSR. AF, atrial fibrillation; RSR, regular sinus rhythm; VEGFR, vascular endothelial growth factor receptor.
the AF group than in the RSR group. The expression of both HIF-1α and HIF-1 oxygen-dependent degradation domain mRNA (data not shown) was similar in the 2 groups (Figure 1). In addition, the HIF-1α and VEGF mRNA levels were correlated (r=0.35, P<0.05). VEGFR-1 mRNA, but not VEGFR-2 mRNA, was upregulated in the atria of the AF group (Figure 2). Western blot analysis revealed that the levels of the VEGF and HIF-1α proteins were significantly higher in the AF group as compared with the RSR group (Figure 3). We also investigated the mRNA and protein expression levels of transforming growth factor (TGF)-β and they were similar in both groups (data not shown). To determine the tissue-specific localization of VEGF and MMP-9, we performed immunofluorescence staining of atrial tissue from the AF and RSR groups, which revealed excessive production of VEGF, HIF-1α, and MMP-9 in the endothelium of the right-atrial appendage arteries of the AF patients. In addition, VEGF and HIF-1α, and VEGF and MMP-9 were all co-localized (Figure 4).

Discussion

Because AF tends to become persistent or chronic as a result of atrial remodeling induced by sustained or repetitive AF, it is necessary to understand the mechanisms of atrial remodeling. A number of studies have demonstrated the changes in electrophysiological properties that occur during AF, and remodeling of ion channels is a probable explanation for these changes. 33–36 The increased expression of gap junctions and activity of MMP–disintegrins cause structural changes in the atrium that contribute to the pathogenesis of sustained AF. 37–41 We previously reported that MMP-9 expression increases in fibrillating human atrial tissue. 25 MMP-9 plays an important role in the degradation and remodeling of damaged tissues, including that of the heart. In the present study, we further demonstrated that the expression of both VEGF and HIF-1α increased in fibrillating atrial tissue. Because VEGF is a potent stimulator of MMP-9 expression, 25,26 excessive MMP-9 expression might be induced by the increased VEGF expression in AF patients.

The fibrillatory state itself consumes more energy, 37 so the high rate of atrial excitation would lead to tissue hypoxia during AF. It has been reported that there is a transient increase in HIF-1α gene expression in the early response of cardiomyocytes to AF. 38 Insufficient oxygen could cause cardiac remodeling and fibrogenesis, resulting in myocardial dysfunction. Cardiac fibrosis could possibly induce an increase in the oxygen diffusion distance from the capillaries to the myocytes, and therefore an imbalance in the myocardial oxygen demand and supply would occur in the atrium. Atrial hypoxia and the HIF pathway may lead to the structural changes of the atria, such as cardiac fibrosis, that may lead to the continuance of AF. 38–40

HIF-1α levels are regulated by proteolysis via an oxygen-sensitive mechanism. The HIF-1α protein undergoes prolyl hydroxylation and is rapidly degraded by the ubiquitin–proteasome system under normoxic conditions. Because prolyl hydroxylation is inhibited under hypoxic conditions, HIF-1α accumulates significantly in the nucleus. 28,30–32 The dimerization of HIF-1α contributes to the transcription of HIF-1-responsive genes, including VEGF, in the nucleus. 28,30–32 In fact, it has been confirmed that the VEGF gene possesses HIF-1-binding sites in its regulatory region. 41,42 Therefore, it is reasonable that the levels of the HIF-1α and VEGF proteins were higher in the present AF group than in the RSR group.
Hypoxia and AF Remodeling

Under the aforementioned circumstances, AF would cause hypoxia by itself. On the other hand, recent studies have indicated that atrial ischemia may induce AF and vice versa. In the present study, there was a higher percentage of coronary artery diseases in the patients in the RSR group than in the AF group (Table). However, the level of expression of VEGF was significantly higher in the AF group than in the RSR group (P<0.05). Additionally, the levels of expression of VEGF mRNA and protein, as well as of HIF-1α protein, were significantly higher in the AF group as compared with the RSR group (P<0.05). Chung et al demonstrated that the plasma levels of VEGF were markedly higher in AF patients than in RSR patients and concluded that AF may cause tissue hypoxia. Gramley et al reported that AF is closely associated with the upregulation of hypoxic and angiogenic markers. The results of the present study also support increases in the levels of expression of VEGF and HIF-1α as a cause of hypoxia induced AF. There is a possibility that the combination of hypoxia and AF could create a vicious cycle that sustains the AF.

In this study, there was no significant difference in the HIF-1α mRNA expression of the 2 patient groups. It is well known that HIF-1α mRNA turnover is high under non-hypoxic conditions. We hypothesized that the HIF-1α mRNA turnover would be transiently low in both groups under hypoxic conditions during cardiac operation. We suggest that the invasive nature of open cardiac surgery would mask any potential difference in the HIF-1α mRNA levels of the 2 groups.

Our result that VEGFR-1 expression increased in the AF group was also consistent with previous studies that have reported that endothelial cells exposed to hypoxia upregulate their VEGFR-1 expression. Wang et al demonstrated that VEGF receptor-1 plays an important mediating role in VEGF-dependent MMP-9 expression in vascular smooth muscle cells. They further confirmed this activation by using PEGF, which is a VEGF receptor-1 specific ligand. Other pathways that increase HIF-1α and VEGF have been reported. Left ventricular myocardial stretch and stress have been reported to induce overexpression of HIF-1α and TGF-β. Others have reported that TGF-β is upregulated in AF, and that TGF-β signaling may regulate VEGF expression. We could not completely rule out the possibility that atrial stretch during AF induced a subsequent overexpression of HIF-1α and VEGF, but the expression of TGF-β mRNA was similar in both groups in this study. Many previous studies have reported TGF-β upregulation in fibrillating atrial tissue; however, this was not observed in our study. In our study, the RSR group subjects included 77% of patients with ischemic heart diseases and 23% with valvular diseases. A possible explanation for this discrepancy is that similar fibrosis might occur in the atria of the patients in both groups.

The interaction between VEGF and the MMPs is well

**Figure 4.** Immunofluorescent staining for VEGF, HIF-1α, and MMP-9. Immunohistochemical analysis showed that MMP-9 exists mainly in the perivascular area of the atria of patients with AF. Double staining revealed that VEGF and HIF-1α, VEGF and MMP-9 are colocalized. Original magnification: Upper panel×200; Lower panel×400. AF, atrial fibrillation; HIF-1α, hypoxia induced transcription factor-1α; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.
established in cancer research. VEGF strongly influences the expression of MMPs, especially MMP-9, which in turn contributes to the bioavailability of VEGF. For example, VEGF can stimulate organ-specific MMP-9 expression and cancer invasion. The anti-VEGF antibody inhibits both MMP-9 expression and tumor invasion into the ovaries. In contrast, VEGF is released from extracellular stores by MMP-9, an event that increases the bioavailability of the angiogenic mitogen. Furthermore, the implication of VEGFR-1 in MMP-9 expression in lung cancer was revealed. Altogether, our results strongly suggest the possibility that MMP-9 upregulation is directed by activation of HIF-1α/VEGF induced by hypoxia. Therefore, not only MMP-9 but also this axis represent a potentially important therapeutic target for AF.

Study Limitations
The characteristics of the AF and RSR groups were quite different and it is difficult to eliminate the influence of different patient backgrounds. The number of patients in this study was small. It was not directly proven whether VEGF/VEGF receptors/HIF-1α induce MMP-9 expression or not, which should be addressed in a future study. However, the results are of value in the study of atrial remodeling in humans and may contribute to further studies of AF; however, study of the molecular mechanisms and animal model experiments.

References


