Proinflammatory gene expression in patients undergoing mitral valve surgery and maze ablation for atrial fibrillation

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ABSTRACT

Objective: It is difficult to achieve rhythm control in patients with long-standing persistent atrial fibrillation (AF). The radiofrequency maze procedure is an effective means in curing AF with a variable recurrence rate depending on patient characteristics and AF duration. In these patients, the characteristics of the atrial substrate have not been well investigated. Because the inflammatory process has been shown to be important in the pathogenesis of AF, we sought to characterize the proinflammatory gene expression in left atria obtained from patients with AF undergoing mitral valve surgery combined with the maze procedure to distinguish the changes associated with AF and its recurrence after the surgical ablation.

Methods: Left atrial appendages from 35 patients receiving mitral valve surgery were used for study. Ten patients had sinus rhythm (SR) and 25 patients had persistent AF for more than 1 year and underwent the maze procedure. Among the AF patients, 13 patients remained in SR (AF-SR) and 12 patients had recurrent AF during the 1-year clinical follow-up (AF-AF). The nCounter Human Inflammation Array (NanoString Technologies, Seattle, Wash) was used for evaluating proinflammatory gene expression. Quantitative polymerase chain reaction, Western blot, and immunohistochemistry were applied for studying messenger RNA and protein expression.

Results: Of 144 expressed proinflammatory genes, the inflammation array analysis revealed that 32 genes were differentially expressed between AF (including AF-SR and AF-AF) and SR. Thirteen genes were differentially expressed between AF-SR and AF-AF. The array and quantitative polymerase chain reaction produced parallel results in analyzing the expression of particular genes. Concordant with the gene expression difference between AF and SR patients, rapid pacing increased the expressions of *SHC1*, *RHOA*, *PDGFA*, and *TRAF2* in HL-1 myocytes, implicating a causative effect of tachyarrhythmia on these genes. Compared with AF-SR, AF-AF expressed more intense oxidative stress, upregulations of collagen, transforming growth factor beta 1, and intranuclear nuclear factor of activated T-cells. Regression analysis showed that increased left atrial diameter was associated with the expression of *RHOA* and *STAT1*.

Gene expression in atrial fibrillation and its recurrence after surgical ablation.

Central Message

Proinflammatory gene expression in patients with AF undergoing maze ablation were characterized.

Perspective

Differential expression profiles of proinflammatory genes were presented between sinus rhythm (SR) and atrial fibrillation (AF) and between maintained SR and recurrent AF after the maze procedure. The identified inflammatory molecules associated with AF and failed surgical ablation may provide clues for finding new potential therapeutic targets to improve AF rhythm control.

See Editorial Commentary page 1683.

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Abbreviations and Acronyms

AF	= atrial fibrillation
AF-AF	= recurrent atrial fibrillation after the maze procedure
AF-SR	= maintained sinus rhythm after the maze procedure
CAD	= coronary artery disease
DHE	= dihydroethidium
IL	= interleukin
LAD	= left atrial diameter
mRNA	= messenger RNA
NFAT	= nuclear factor of activated T-cells
NF- κ B	= Nuclear factor- κ B
qPCR	= quantitative polymerase chain reaction
SR	= sinus rhythm
TGF- β 1	= transforming growth factor- β 1

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Conclusions: Differential expression profiles of proinflammatory genes were presented between SR and AF and between maintained SR and recurrent AF after the maze procedure. The identified inflammatory molecules associated with AF and failed surgical ablation may provide clues for developing new potential therapeutic targets to improve AF rhythm control. (J Thorac Cardiovasc Surg 2016;151:1673-82)

Atrial fibrillation (AF) is the most common sustained arrhythmia. It is associated with increased risks of worsening heart failure, ischemic stroke, and death. As AF persists over time, atria substrate remodeling becomes more severe and rhythm control becomes more difficult (AF begets AF).¹⁻³ Although major progress in AF rhythm control has been made mainly by catheter ablation during the past 20 years, AF recurrence is still a substantial clinical issue. In patients with longstanding (>1 year) persistent AF, AF recurrence rate has been reported to be more than 50% 1 year after single catheter ablation, which is quite disappointing. Clinical and animal studies have suggested that prolonged AF substantially results in more severe and frequently irreversible atrial remodeling regarding both gene expression changes and histologic features.⁴⁻⁶

Among patients undergoing mitral valve surgery, AF is associated with reduced survival and increased risk of stroke. Surgical ablation of AF is an effective treatment of rhythm control. The Cox-maze III surgical operation is the gold standard for surgical treatment of AF with a reported AF cure rate of 75% to 90%,^{7,8} in contrast to the modified maze procedure, with a reported AF cure rate ranging from 44% to 90%.⁹⁻¹⁴ Several clinical factors have been demonstrated to be associated with AF recurrence after the maze procedure, including the preoperative duration of AF, atrial size by echocardiography, and AF wave characteristics obtained during the cardiac surgery.¹⁵ However, the atrial substrate in these patients has not been well investigated. The maze procedure is highly effective in treating AF, but the recurrence rate is still high in patients with markedly dilated atria. Enlarged atria are thought to be associated with more severe atrial remodeling, which increases the difficulties in treating AF. It could be helpful to improve the treatment if we find any molecular targets that can help distinguish failed and successful maze surgery in these patients with markedly dilated atria. Inflammation is a response that involves immune cells, blood vessels, and molecular mediators. The inflammatory process is widely involved in many human diseases, including cardiovascular disease. Numerous studies have shown mediators of the inflammatory response alter atrial electrophysiology and worsen structural substrates, thereby leading to increased AF vulnerability and refractoriness.¹⁶⁻¹⁸ This evidence includes elevated serum levels of inflammatory biomarkers, including C-reactive protein, tumor necrosis marker, interleukin (IL)-2, IL-6, and monocyte chemoattractant protein-1 in AF patients and upregulation of inflammatory mediators in the atria of AF patients and animal models of AF. Inflammation links to various pathologic processes, such as oxidative stress; electrical and calcium handling abnormalities; and fibrosis, which promotes atrial substrate remodeling. An improved understanding of the underlying pathophysiology should help improve the management options for AF. The inflammatory process is broadly diverse and contains numerous signaling pathways and mediators. In the past the inflammation-related studies in AF were mostly selective and have targeted genes or proteins of interest. Here we intended to find any potential targets and therefore chose a commercial set containing extensive inflammation-related targets to screen a panel of genes that are likely involved in atrial remodeling. Therefore, in this study, we sought to characterize gene expression profiles of mediators and transcription factors relevant to inflammation in the atria among patients with preoperative sinus rhythm (SR), maintained SR, and recurrent AF at 1 year after the maze procedure in patients with mitral valve disease undergoing

valvular surgery. Our data reveal that several transcriptional factors and mediators are differentially expressed among the 3 groups.

MATERIALS AND METHODS

Maze Ablation and Human Samples

From September 2008 to August 2013, 261 patients with severe mitral valve disease and persistent AF with a duration known to be longer than 12 months underwent mitral valve surgery and maze procedure. Patients were operated on by a single surgeon with technical unity and the first 10 cases were excluded to avoid learning curve bias. Our routine approach was standard full sternotomy with bicaval cannulations, and AF ablation lesion sets were similar to traditional Cox-maze III, except most cut-and-sew lesions were replaced by the irrigated bipolar radiofrequency ablation device (Medtronic, Minneapolis, Minn) to create long continuous transmural lesions. Separated pulmonary vein isolation with 2 connecting lesions of both superior and inferior pulmonary veins were applied twice in both sides to confirm the complete ablation. Additional cryoablation of the mitral isthmus was applied with a cryoprobe (-70°C for 2 minutes). Biatrial ablation was applied in most cases and left atrial ablation alone was applied solely in elderly patients with isolated aortic valve replacement. The left atrial appendage was closed from inside with 4-0 Prolene (Ethicon, Inc, Cincinnati, Ohio) double running sutures and resected from outside for specimen analysis. The ligament of Marshall was divided during left pulmonary vein isolation and bilateral epicardial fats over the interatrial groove, composed of ganglionic plexi, were also resected. Left atrial volume reduction for markedly enlarged left atrium, dimensions >60 mm by preoperative echocardiography, was carried out with applications of posterior wall between the inferior pulmonary vein and mitral isthmus. Of these patients, sustained sinus rhythm, including sinus rhythm or an atrial-based paced rhythm, was present in 78% at 1 year. Between January 2011 and August 2013, 42 patients who agreed to participate in the study were enrolled and atrial specimens were obtained from these patients. These patients had no evidence of atrioventricular node dysfunction before surgery. Among these patients, 12 had recurrent AF and the others maintained sinus rhythm during 1-year follow-up. The mean $\pm$ standard deviation left atrial diameter (LAD) by echocardiography was 58 ± 10.1 mm for patients with recurrent AF and 48.3 ± 13.0 mm for patients with maintained SR, respectively ($P = .065$). AF recurrence rate is high in patients with markedly dilated atria. In this study we compared patients with more dilated atria with maintained SR ($n = 13$) and all enrolled patients with recurrent AF. Another 10 samples of left atrial appendage were obtained from patients with mitral valve disease and SR during the valvular surgery (SR group). Antiarrhythmic drugs were used by 6 patients of the maintained SR after the maze procedure group (AF-SR) who were free of AF. During follow-up at 1 year after the valvular surgery, cardiac echography showed no significant recurrent mitral regurgitation or stenosis in either the recurrent AF after the maze procedure (AF-AF) or AF-SR groups. The research protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Chang-Gung Medical Foundation Institutional Review Board. Written informed consent was obtained from all participating patients.

Proinflammatory Gene Expression Analysis

Total RNA was isolated from left atrial appendages with the TRIzol reagent (Invitrogen, Carlsbad, Calif) and was further purified and concentrated by using the WelPrep tissue RNA kit (Welgene, Taipei, Taiwan). The quality and quantity of total RNA were analyzed by using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, Calif). Gene expression profiles in tissue RNA were analyzed with an nCounter Human Inflammation kit (NanoString Technologies, Seattle, Wash) following the manufacturer's protocols.

Quantitative Polymerase Chain Reaction

Total RNA was extracted by using the TRIzol reagent, and quantitative polymerase chain reaction (qPCR) was performed. The oligonucleotide sequences used are given in Table E1. Glyceraldehyde 3-phosphate dehydrogenase messenger RNA (mRNA) was used as the internal control.

Confocal Immunohistochemistry

Immunohistochemistry and quantitative assessment were carried out as described previously.¹⁹ Immunohistochemical analyses were performed using confocal microscopy (Leica TCSSP2, Mannheim, Germany) with collagen-1, transforming growth factor- β 1 (TGF- β 1), nuclear factor of activated T-cells cytoplasmic 3 (NFATc3), and vimentin (Sigma-Aldrich, St Louis, Mo) as primary antibodies, followed by fluorescein isothiocyanate (green) or Cy3 (red; Chemicon, Temecula, Calif) conjugated secondary antibodies. Nuclei were visualized by 40,6-diamidino-2-phenylindole staining. The expression of target proteins was calculated as protein-occupied area in the tissue divided by the nucleus area. For NFATc3, only the area within the nuclei was estimated. For each analysis, at least 5 random fields were chosen with >30 observations in each. Oxidative stress from reactive oxidative species in the tissues was measured with the fluorescent dye (dihydroethidium [DHE]) and was detected by using confocal microscopy. Samples were preincubated with or without 10 mmol/L DHE for 30 minutes. DHE was excited at 543 nm with an argon laser, and emission at 590 nm was recorded. Two-dimensional images (512×512 pixels) were acquired and analyzed with MetaMorph software (Universal Imaging Corp, West Chester, Pa).

Western Blotting

Proteins were extracted and processed as described previously.²⁰ Briefly, specimens of the left atrial appendage were lysed with lysis buffer. After proteins were separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, membranes were incubated overnight at 4°C with primary antibodies against RHOA, PDGFA, phospho-STAT1 (Millipore, Temecula, Calif), SHC1, TRAF2, CCL4, IL15, total STAT1, NFATC3 (Abcam, Cambridge, Mass), GAPDH, or tubulin (Santa Cruz, Dallas, Tex). Signals were detected by using electrochemiluminescence and were quantified using densitometry. Immunoreactive signal bands were in the linear range and expressed relative to GAPDH or tubulin. The software Image Gauge (Fuji Film, Tokyo, Japan) was used for the quantitative evaluation of the protein expression.

Cell Culture and Tachypacing

HL-1 atrial myocytes were maintained in Claycomb medium and subjected to field stimulation as described previously.¹⁹ HL-1 cells ($\geq 1 \times 10^6$ cells) on 4-well rectangular dishes (Nunc, Rochester, NY) were placed into C-Dish 100 TM-Culture Dishes (IonOptix, Milton, Mass). HL-1 cells were then subjected to field stimulation with 10-ms stimuli of 40-V intensity at 1, 2, and 4 Hz frequency for 24 hours (1.5-V/cm field strength; C-Pace EP culture pacer, IonOptix). The capture efficiency $>90\%$ was confirmed by microscopic examination.

Statistical Analysis

For patient characteristics, mean $\pm$ standard deviation for continuous variables and count and percent for categorical variables are presented, respectively. Fisher exact test was used for category comparisons between groups. The data in the figures are presented with box-and-whisker plots. Unpaired Student t test and 1-way analysis of variance with post hoc Tukey tests were applied for the 2 groups and multiple comparisons, respectively. For proinflammatory gene expression analysis, the data were normalized to the expression of housekeeping genes like *GAPDH* for each set and the significant difference between 2 groups was considered with a false

TABLE 1. Demographic and clinical features of patients with sinus rhythm (SR), maintained SR (AF-SR) and recurrent atrial fibrillation (AF-AF) after undergoing a maze procedure

Feature	SR (n = 10)	AF-SR (n = 13)	AF-AF (n = 12)
Demographic			
Age (y)	62.5 ± 14.6	56.6 ± 11.6	58 ± 10.1
Sex (M:F)	5:5	5:8	8:4
Laboratory			
White blood cell count (1000/ μ L)	6.6 ± 1.8	7.1 ± 1.9	6.9 ± 2.1
Clinical			
Ejection fraction (%)	48.4 ± 24.3	57.15 ± 14.9	65.4 ± 10.3
Left arterial diameter (mm)	46.3 ± 9	58.7 ± 13.8*	56 ± 10.9
Rheumatic heart disease	0	6*	5*
Hypertension	6	4	2
Diabetes mellitus	5	4	3
Coronary artery disease	6	2*	1*
Medication			
Aspirin	6	2*	1*

Values are presented as mean $\pm$ standard deviation or n. SR, Sinus rhythm; AF-SR, maintained sinus rhythm after the maze procedure; AF-AF, recurrent atrial fibrillation after the maze procedure. * $P < .05$ versus sinus rhythm.

discovery rate $<5\%$. Pearson correlation was used for correlation between expressions of proinflammatory gene and left atrial diameter.

RESULTS

Patient Characteristics and Proinflammatory Gene Expression Analysis

The clinical characteristics of patients are listed in Table 1. The age and left ventricular ejection fraction

were not significantly different among SR, AF-SR, and AF-AF groups. The LAD as measured by 2-dimensional echocardiography was significantly smaller in SR patients compared with AF patients (including AF-SR and AF-AF). The LAD was not significantly different between AF-SR and AF-AF groups. There was no difference in sex between SR and AF patients, but there were more women enrolled in the AF-SR group compared with the AF-AF group. In patients with AF, 6 of 13 in AF-SR and 5 of 12 in AF-AF had rheumatic heart disease presenting with significant mitral stenosis (P value not significant). In SR, more patients had coronary artery disease (CAD), hypertension, and diabetes mellitus compared with AF-SR and AF-AF.

Of the 184-probe sets, 144 genes were expressed at detectable levels (Table E2) in these samples. Among these, 32 genes were differentially expressed between SR and AF (including group AF-AF and AF-SR) (Figure 1). Fourteen genes were significantly upregulated in AF compared with SR, including *HSPB1*, *STAT1*, and *PDGFA*. Eighteen genes were significantly downregulated in AF, including *IL1B* and *CXCL6*. Thirteen genes were significantly differently expressed between group AF-AF and AF-SR (Figure 1). Ten genes were upregulated in AF-AF compared with AF-SR, including *CCL4* and *IL15*, whereas 3 of them were downregulated in AF-AF such as *STAT1*.

In AF-AF group, 7 patients had recurrence of AF within the first month after the maze procedure. Recurrent AF was noted for the other 3, 1 patient at 3, 6, and 7 months, respectively. There was no association between the

FIGURE 1. Significant differentially expressed proinflammatory genes in left atrial appendages (A) between sinus rhythm (SR) and atrial fibrillation (AF) and (B) between recurrent AF after the maze procedure (AF-AF) and maintained SR after the maze procedure (AF-SR). The results are presented as log 2 (SR/AF) and log 2 (AF-SR/AF-AF), respectively. LA, Left atria.

FIGURE 2. Comparison between quantitative polymerase chain reaction (*qPCR*) and nCounter Human Inflammation Array (NanoString Technologies, Seattle, Wash) (A) between atrial fibrillation (*AF*) and sinus rhythm (*SR*) and (B) between recurrent *AF* after the maze procedure (*AF-AF*) and maintained *SR* after the maze procedure (*AF-SR*). Data are expressed as log 2 fold change. Genes were selected by virtue of significant differences between groups in inflammation array analysis. The results were presented as log 2 (*SR/AF*) and log 2 (*AF-SR/AF-AF*), respectively. For *qPCR*, data are presented as box-and-whisker plot. N = 5 to 7 samples for each gene. *LA*, Left atria.

expression level of proinflammatory genes and AF-free duration in the group.

qPCR

To validate the inflammation array analysis, genes of interests were selected for *qPCR* analysis, which showed that the array and *qPCR* analyses produced parallel results with respect to the up- and downregulation of particular genes, but with modest differences in the magnitude of specific changes (Figure 2, A and B).

Protein Expression

To evaluate whether the gene expression difference at transcriptional level was reflected on translational level, the expression of several genes were analyzed with Western blot. Consistent with the findings at the mRNA levels, we found that the expressions of *SHC1*, *RHOA*, *PDGFA*, and *TRAF2* were significantly increased in *AF* compared with *SR* (Figure 3, A, and Figure E1, A). Figure 3, B, and Figure E1, B, show the expressions of *CCL4* and *IL15* were significantly increased in *AF-AF* compared with *AF-SR*. The expression of phospho-*STAT1* was significantly reduced in *AF-AF* compared with *AF-SR*, whereas the total form of *STAT1* was not different between the 2 groups. Although at the mRNA level both nuclear factor of activated T-cells, cytoplasmic (NFAT) and RAC1-related C3 botulinum toxin substrate 1 were differentially expressed between

AF-AF and *AF-SR*, the expressions of both at the protein level were not significantly different.

Ultrastructural Remodeling

To evaluate if cardiac rhythm (*AF* vs *SR*) and *AF* refractoriness to the maze procedure are associated with the severity of atrial substrate remodeling, the expression of collagen I, TGF- β 1, NFATc3, and oxidative stress was evaluated by immunofluorescence. Figure 4 and Figure E2 showed that the expression of collagen, TGF- β 1, and oxidative stress was significantly increased in both *AF-AF* and *AF-SR* compared with *SR*. Moreover, the expressions of TGF- β 1 and oxidative stress were significantly higher in the atria of *AF-AF* compared with *AF-SR*, which implies that *AF* recurrence after surgical ablation is associated with more severe atrial substrate remodeling. NFAT is a nuclear transcription factor that has been shown to be involved in *AF*-related electrical and structural remodeling. Dephosphorylated NFAT was translocated into nucleus and module downstream gene expression.²¹ Consistent with previous findings, we observed that the expression of intranuclear NFATc3 was more abundant in both *AF-AF* and *AF-SR* compared with *SR*; furthermore, its expression was also significantly higher in *AF-AF* compared with *AF-SR*, suggesting that NFAT activation is associated with more severe *AF* remodeling and that it may contribute to *AF* refractoriness in the surgical ablation.

ACQ

FIGURE 3. A, Western blot analysis of *SHC1*, *RHOA*, *PDGFA*, and *TRAF2* between sinus rhythm (SR) and atrial fibrillation (AF). B, Western blot analysis of *CCL4*, *IL15*, phospho- and total *STAT1*, *RAC1*, and *NFATc3* between recurrent AF after the maze procedure (AF-AF) and maintained SR after the maze procedure (AF-SR). The relative expression levels of proteins corresponding to *GAPDH* were quantified by densitometry and normalized to A, SR and B, AF-SR, respectively, which was set at 1.0. N = 3 to 5 for each group. *P < .05.

Tachypacing-Induced Gene Expression

To determine if tachycardia have a causal relationship with the expression of the AF-associated remodeled genes, HL-1 myocytes were subjected to external electric activation at 1, 2, and 4 Hz for 24 hours. Figure 5 and Figure E3 indicated that the expression of *PDGFA*, *RHOA*, and *TRAF2* at protein level was significantly increased, whereas *SHC1* was reduced, by electric pacing in a frequency-dependent manner. The changes were

FIGURE 4. Confocal immunohistochemistry analysis of reactive oxidative stress (*ROS*), transforming growth factor beta 1 (*TGF-β1*), collagen, and nuclear factor of activated T-cells cytoplasmic 3 (*NFATc3*) between sinus rhythm (SR), maintained sinus rhythm after maze procedure (AF-SR) and recurrent atrial fibrillation after maze procedure (AF-AF). The relative intensity is normalized to SR, which is set at 1.0. N = 3 to 5 samples for each group. *Versus SR. #Versus AF-SR (P < .05).

FIGURE 5. Activation of HL-1 atrial myocyte regulated the expression of *SHC1*, *RHOA*, *PDGF-A*, and *TRAF2* at protein level at a frequency-dependent manner. The relative expression levels of proteins corresponding to *GAPDH* were quantified by densitometry and normalized to 1-Hz pacing, which was set at 1.0. N = 4 for each group. *P < .05 versus 1 Hz.

similar to the findings in human AF versus SR, which implies that AF may have a causal relationship with the expression of these genes.

Correlation Between Proinflammatory Genes and LAD

The severity of atrial remodeling may be reflected in the degree of atrial dilatation. Because previous studies have shown that the atrial size is an independent factor predicting AF recurrence after the maze procedure,^{22,23} we evaluated the relationship between the expression of proinflammatory genes and the LAD by 2-dimensional echocardiography based on these specimens. Figure 6 shows that the expression of *RHOA* and *STAT1* significantly correlated with the LAD. Consistent with prior research, our findings suggest that these proinflammatory molecules were likely involved in adverse remodeling associated with atrial dilation.

Correlation Between Proinflammatory Genes and AF Recurrence After the Maze Procedure

To evaluate if the expressions of proinflammatory genes help distinguish AF-AF and AF-SR in patients with markedly dilated atria, we performed the receiver operator curve analysis. The highest area under the curve is 0.82 for *PTK2* as shown in Figure 7. The area under the curve is 0.763 for all of *NFATc3*, *RAC1*, and *CCL4* and 0.79 for *STAT1*, respectively.

DISCUSSION

Main Findings

Through an unadjusted screening of inflammation-related genes, we found a differential gene expression profile in the left atria after the surgical ablation of SR versus AF and maintained SR versus AF recurrence. We further compared the characteristics of the atria between maintained SR versus AF recurrence after the surgical ablation and demonstrated that more intense structural remodeling in the atria of AF-AF compared with AF-SR,

FIGURE 6. Regression analysis between left atrial diameter (LAD) and (A) *RHOA* and (B) *STAT1*. SR, Sinus rhythm; AF-SR, maintained sinus rhythm after maze procedure; AF-AF, AF recurrence after maze procedure.

further implying that severe structural remodeling as well as gene expression changes may underlie the failure to maintain sinus rhythm after surgical ablation. The results indicate that several inflammation-related transcription factors and mediators may present as potential targets in attenuating atrial remodeling by AF and improving AF therapy.

Like catheter ablation or use of antiarrhythmic drugs in treating AF, the AF-free rate after the maze surgery is significantly reduced by the enlarged atria. It is generally agreed that a more dilated atria is associated with more severe atrial remodeling, which makes it difficult to get rhythm control. Better understanding about differential gene expressions between maintained SR and recurrent AF in the patients with markedly dilated atria may help develop strategies to reduce the recurrence rate after the ablation. In this study we found beyond the effect of atrial size, AF recurrence after the maze surgery is associated with more severe atrial remodeling, presented as more

intense oxidative stress, upregulations of collagen, TGF- β 1, and differential expression profiles of proinflammatory genes.

Inflammation-Related Gene Remodeling in AF Versus SR

Our data revealed that several genes were differentially expressed between AF and SR. *STAT1* is a proinflammatory transcription factor downstream to *AT1R* and *PDGFR*. In a pig atria tachypacing-induced AF model, both *STAT1* and *RAC1* were upregulated and activated, which means that its pro-AF role seems to involve *RAC1* signaling.²⁴ Echoing findings from the animal study, our data showed that both the expression and activity of *STAT1* were increased in AF compared with SR. Among these genes altered in human AF, as revealed by the array, we demonstrated that in cultured HL-1 atrial myocytes, rapid cellular activation by electric pacing increased the expressions of *RHOA*, *PDGFA*, and *TRAF2*, whereas the expression of *SHC1* was decreased by rapid pacing, suggesting that tachycardia exerts a causal relationship with the expression of these inflammation-related genes. Research has posited *RHOA* and *PDGFA* as proinflammatory genes that are associated with AF.^{25,26} The findings from these studies imply that these AF-upregulated genes may therefore participate in the process of AF begets AF in atrial substrate remodeling. TRAF2 is a scaffolding protein common to tumor necrosis factor receptors 1 and 2, and a recent study has demonstrated that TRAF2-mediated cytoprotective effects of tumor necrosis factor in the heart.²⁷ This means that the upregulation of TRAF2 in human heart could be protective against AF remodeling. The role of *SHC1*, on the other hand, is more conflicting. *SHC1* encodes the protein p66Shc, which has been shown to have pro-oxidant and proapoptotic activity and to be implicated in the aging process. In contrast, a recent study provided

FIGURE 7. Receiver operator curve analysis of *PTK2* in recurrent atrial fibrillation after the maze procedure and maintained sinus rhythm after the maze procedure. AUC, Area under the curve.

evidence that genetic deletion of p66Shc led to increased myocardial infarction in response to short-term ischemia and reperfusion, which means that activation of p66Shc protein seems to represent a protective strategy to prevent ischemic and reperfusion myocardial injury.²⁸ The role of *SHC1* in AF is still largely unclear.

HSPB1 is small heat shock protein. Overexpression of HSPB1 has been shown to be protective against tachycardia-induced remodeling by attenuation of the *RHOA* GTPase pathway in in vivo and in vitro models.^{26,29} It has been shown that the induction of heat shock proteins attenuate atrial tachycardia-induced electrical (reduction of expressions of L-type calcium channels) and structural (increased collagen accumulation in the atria) remodeling. Its upregulation in AF may represent a compensatory mechanism against AF-related remodeling despite that it cannot keep patients in SR in the conditions of severe volume and/or pressure overload due to mitral valve dysfunction.

The characteristics of patients in SR group are different from patients in the AF-SR and AF-AF groups. All the patients in the SR group were diagnosed with nonrheumatic heart disease and more of them had coexisting CAD, diabetes mellitus, and hypertension. Therefore it should be emphasized that the differential gene expressions between SR and AF may not only contribute to cardiac rhythm but also the underlying disease (rheumatic and nonrheumatic). Despite this, we found genes like *SHC1*, *RHOA*, and *PDGFA* were upregulated by tachycardia at a cellular tachypacing model of HL-1 cells, which implies a causative relationship from tachycardia to gene changes potentially present.

In the study more patients in the SR group had CAD and therefore were taking aspirin, an anti-inflammatory agent, which may also contribute to the differential proinflammatory gene expressions between SR and AF.

Inflammation-Related Gene Remodeling in Successful Versus Failed Rhythm Control by the Maze Procedure

Our data firstly showed that several genes were differentially expressed or activated in the atria between patients with maintained SR or recurrent AF after the maze procedure.

NFAT is a nuclear transcription factor downstream to calcineurin and calcium ion/calmodulin. Activated calcineurin dephosphorylates NFAT and causes its translocation to the nucleus. Numerous studies have reported increased cardiac calcineurin activity and NFAT nuclear translocation in human failing and hypertrophied hearts as well as in arrhythmia animal models.^{30,31} It has been shown in canine atrial myocytes that NFAT is activated by rapid electrical stimulation mimicking the condition of atrial tachycardia, which mediates

tachypacing-induced Cav1.2 downregulation and explains the electric mechanisms of AF begets AF in respect of decreased L-type calcium current in AF.²¹ The same group proposed in another study that the atrial fibroblasts were also activated in an AF-promoting dog model, in which NFAT in the fibroblasts were activated as well and also played a profibrotic role in AF.³² Consistent with the previous studies, we observed higher expression of nuclear NFATc3 and increased oxidative stress in patients with recurrent AF after the maze procedure, indicating that NFAT activation is likely associated more advanced atrial remodeling as well as oxidative stress, which may make it more difficult for rhythm control to be achieved.

Several proinflammatory mediators were shown to be upregulated in AF-AF compared with AF-SR. For example, both *CCL4* and *IL15* are cytokines upregulated during the inflammatory response and may be involved in recruitment of neutrophils, and both of which may contribute to inflammatory cell infiltration in the atria and worsen atria remodeling in AF. Our results indicated that both *CCL4* and *IL15* were more highly expressed in the group of failed rhythm control, probably reflecting severe inflammation in AF. At present, their roles in the heart remain to be elucidated.

Previous Studies

Few research groups have investigated the association between atrial substrate remodeling and the outcome of surgical ablation. Saito and colleagues³³ examined the morphologic changes in atrial appendages removed during the maze procedure. They found patients with recurrent AF have more myocyte hypertrophy, nuclear enlargement, bizarre nuclei, and interstitial fibrosis compared with those patients who still maintained SR. Castoguy and colleagues³⁴ found both myocyte hypertrophy and interstitial fibrosis were more often seen in the atria of patients with recurrent AF. Wang and colleagues³⁵ found that the expression of matrix metalloproteinase-2 and collagen I were significantly higher in the atria from patients with recurrent AF compared with the patients with maintained SR. Our findings revealed that a number of proinflammatory genes differentially expressed between AF and SR and between AF-AF and AF-SR. Consistent with our previous studies in AF remodeling,^{20,36} we showed that increased oxidative stress, expression of TGF- β 1, and activation of NFAT may underlie the more severe changes of substrate remodeling changes in the atria that is refractory to surgical ablation. The data may provide a database and clues to search for potential therapeutic targets in preventing atrial remodeling progression and managing AF.

Nuclear factor- κ B (NF- κ B) is a nuclear transcription factor coupling redox state to changes in gene transcriptional regulation. It has been shown that NF- κ B was associated

with AF, and increased oxidative stress reduced the expression of cardiac sodium channel via NF- κ B.³⁷ However, we found that NF- κ B was downregulated in AF compared with SR (Figure 1, A). Another example is *STAT1*, which is thought to be pro-oxidative and has been shown to contribute to AF in the literature. Our results indicate that although *STAT1* was upregulated in AF, its expression was higher in AF-SR compared with AF-AF, which is not consistent with our findings of the more severe remodeling in AF-AF atria. We believe that the interplay between these mediators and AF and its underlying disease is complex and cannot be simply interpreted according to our observations in this study. More detailed studies are required to explore their relationship with AF pathogenesis.

The inflammatory process is broadly diverse and contains numerous signaling pathways and mediators. Considering age as well as underlying systemic and cardiovascular disease in humans and the complex interaction between them, it is likely that not all the inflammatory processes are equally or concordantly involved in a diseased atria. This may explain why we revealed only a portion of proinflammatory genes were differentially expressed among the genes screened in this study.

Study Limitations

We applied the cryoablation, therefore AF recurrence could be contributed by incomplete transmural lesion. We minimized the confounding possibility by limited duration to enroll the patients, employing a single operator, and comparing patients with atria dilated to a similar degree. Further studies are needed to verify our hypotheses that more severe atrial remodeling and differential gene changes are associated with recurrent AF in patients with markedly dilated atria. AF is a complex disease, involving a number of etiologic factors and arrhythmia mechanisms.³⁸ The generalizability of the results of this study is limited by the relatively small sample size of patients. When considering differences in age, stages of heart function, and drug therapy in human AF, the interpatient variability may be a potentially significant confounding factor. Although our findings cannot represent the whole profile of AF in humans based on the limited patient number, this study includes patients with severe mitral valve dysfunction and longstanding persistent AF, which represent a subgroup of AF, from the view of rhythm control, being among the most severe forms. Our findings are thus valuable to provide potential therapeutic targets when we wish to cure difficult AF. We did not approach the systemic inflammation of the study patients, which may influence on the properties of the atria in addition to AF. Lastly, we did not report the duration of AF because it was not available or clear in some cases. However, in this study all of the cases of AF were more than 1 year's duration.

CONCLUSIONS

Our study provides new data distinguishing AF and SR and successful and failed (or difficult) restoration of sinus rhythm after maze surgery. These may provide a clue for further data mining and hypothesis generation in AF, which may help improve current modalities in treating AF.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: atrial fibrillation, maze, NFAT, inflammation

FIGURE E1. A, Representative examples of Western blot of *SHC1*, *RHOA*, *PDGFA*, and *TRAF2* between sinus rhythm (*SR*) and atrial fibrillation (*AF*) and B, Representative examples of Western blot of *CCL4*, *IL15*, phospho-, and total *STAT1*, *RAC1*, and nuclear factor of activated T-cells cytoplasmic 3 (*NFATc3*) between recurrent atrial fibrillation after the maze procedure (*AF-AF*) and maintained sinus rhythm after maze procedure (*AF-SR*).

FIGURE E2. Representative examples of confocal immunohistochemistry of reactive oxidative stress (*ROS*), transforming growth factor beta 1 (*TGF-β1*), collagen, and nuclear factor of activated T-cells cytoplasmic 3 (*NFATc3*) between sinus rhythm (*SR*), maintained sinus rhythm after maze procedure (*AF-SR*) and recurrent atrial fibrillation after maze procedure (*AF-AF*).

FIGURE E3. Representative examples of Western blot of *SHC1*, *RHOA*, *PDGF-A*, and *TRAF2* in HL-1 atrial myocyte after pacing.

TABLE E1. List of primers applied by quantitative polymerase chain reaction in left atrial appendage

Gene	Genebank ID	Primer sequence
CCL4	NM_002984.2	Forward: 5'-GCTTCCTCGCAACTTTGTGG-3' Reverse: 5'-TCACTGGGATCAGCACAGAC-3'
CFB	NM_001710	Forward: 5'-ACAACAGAAGCGGAAGAT-3' Reverse: 5'-ACCATAACTTGCCACCTT-3'
CFD	NM_001928	Forward: 5'-GCGACTCCATCTCTACAA-3' Reverse: 5'-CTGCGTTCAAGTCATCCT-3'
CXCL6	NM_002993.3	Forward: 5'-CCTCTCTTGACCACTATGAG-3' Reverse: 5'-GTTTTGGGGTTTACTCTCAG-3'
GNAS	NM_080425	Forward: 5'-CAACTTACTCCGCAACTT-3' Reverse: 5'-ACCTTCTTGACCTTGAGA-3'
HRAS	NM_005343.2	Forward: 5'-ACCATTTTGTGGACGAATAC-3' Reverse: 5'-AAGACTTGGTGTGTGATG-3'
HSPB1	NM_001540	Forward: 5'-AAACGGGTCATTGCCATTA-3' Reverse: 5'-CGCTTTTATGCTCCTCCA-3'
IL10	NM_000572.2	Forward: 5'-TCCAGTGTCTCGGAGGGATT-3' Reverse: 5'-TGGCCACAGCTTTCAAGAATG-3'
IL15	NM_172174	Forward: 5'-CTACTGGCTCTCATTGACT-3' Reverse: 5'-GAGGATTACATATAGTTCTTCTT-3'
IL18	NM_001562.2	Forward: 5'-CCTTTAAGGAAATGAATCCTCC-3' Reverse: 5'-CATCTTATTATCATGTCCTGGG-3'
IL1B	NM_000576	Forward: 5'-TACATCAGCACCTCTCAAG-3' Reverse: 5'-ATTCAGCACAGGACTCTC-3'
MEF2A	NM_005587	Forward: 5'-GAACTACTTACAGGCACATT-3' Reverse: 5'-ACGAAGGAATCACTTAACAC-3'
PDGFA	NM_002607.5	Forward: 5'-CGCAGGAAGCAGGAATGTCT-3' Reverse: 5'-ACAGCGAGGAGGTGTGGTTT-3'
PPP1R12B	NM_032103.1	Forward: 5'-GGGCCAGAAGTAGCTTGAC-3' Reverse: 5'-TGTTCCGGGTTTCAGTAGAGT-3'
RHOA	NM_001664.2	Forward: 5'-GCTGGGCTAAGTAAATAGGAATTGG-3' Reverse: 5'-GTCCAGGTGAGACAGGTTATGC-3'
SHC1	NM_183001.4	Forward: 5'-GCGAGACTGAGAGGCAGAA-3' Reverse: 5'-CCGGGCCTGTAGAAGGTACTC-3'
STAT1	NM_007315.2	Forward: 5'-TGCTGAGGTTTAGCTGTCTAGTTC-3' Reverse: 5'-GTCTCTTGGCTAGTGCAGTAAA-3'

TABLE E2. List of genes detected by nCounter Human Inflammation Array (NanoString Technologies, Seattle, Wash) in left atrial appendage

Gene symbol	GeneBank Accession	Gene name
Cytokine		
CCL11	NM_002986.2	chemokine (C-C motif) ligand 11
CCL13	NM_005408.2	chemokine (C-C motif) ligand 13
CCL19	NM_006274.2	chemokine (C-C motif) ligand 19
CCL2	NM_002982.3	chemokine (C-C motif) ligand 2
CCL21	NM_002989.2	chemokine (C-C motif) ligand 21
CCL24	NM_002991.2	chemokine (C-C motif) ligand 24
CCL3	NM_002983.2	chemokine (C-C motif) ligand 3
CCL4	NM_002984.2	chemokine (C-C motif) ligand 4
CCL5	NM_002985.2	chemokine (C-C motif) ligand 5
CCL8	NM_005623.2	chemokine (C-C motif) ligand 8
CXCL1	NM_001511.1	chemokine (C-X-C motif) ligand 1
CXCL10	NM_001565.1	chemokine (C-X-C motif) ligand 10
CXCL2	NM_002089.1	chemokine (C-X-C motif) ligand 2
CXCL3	NM_002090.2	chemokine (C-X-C motif) ligand 3
CXCL6	NM_002993.3	chemokine (C-X-C motif) ligand 6
CXCL9	NM_002416.1	chemokine (C-X-C motif) ligand 9
IL1B	NM_000576.2	interleukin 1, beta
IL10	NM_000572.2	interleukin 10
IL15	NM_172174.1	interleukin 15
IL18	NM_001562.2	interleukin 18
IL6	NM_000600.1	interleukin 6
IL7	NM_000880.2	interleukin 7
IL8	NM_000584.2	interleukin 8
LTB	NM_002341.1	lymphotoxin beta
PDGFA	NM_002607.5	platelet-derived growth factor alpha polypeptide
TGFB1	NM_000660.3	transforming growth factor, beta 1
TGFB2	NM_003238.2	transforming growth factor, beta 2
TGFB3	NM_003239.2	transforming growth factor, beta 3
TNF	NM_000594.2	tumor necrosis factor
Receptor		
CD4	NM_000616.3	CD4 molecule
CD40	NM_001250.4	CD40 molecule, TNF receptor superfamily member 5
CD55	NM_000574.3	CD55 molecule, decay accelerating factor for complement
CDC42	NM_001039802.1	cell division cycle 42
CCR1	NM_001295.2	chemokine (C-C motif) receptor 1
CXCR4	NM_003467.2	chemokine (C-X-C motif) receptor 4
C3AR1	NM_004054.2	complement component 3a receptor 1
DAXX	NM_001350.3	death-domain associated protein
GRB2	NM_203506.2	growth factor receptor-bound protein 2
HLA-DRB1	NM_002124.1	major histocompatibility complex, class II, DR beta 1
IL1RAP	NM_002182.2	interleukin 1 receptor accessory protein
IL1RN	NM_173842.1	interleukin 1 receptor antagonist
IL1R1	NM_000877.2	interleukin 1 receptor, type I
IL10RB	NM_000628.3	interleukin 10 receptor, beta
IL18RAP	NM_003853.2	interleukin 18 receptor accessory protein
IL6R	NM_000565.2	interleukin 6 receptor
IL8RA	NM_000634.2	interleukin 8 receptor, alpha
IL8RB	NM_001557.2	interleukin 8 receptor, beta
LY96	NM_015364.2	lymphocyte antigen 96
HLA-DRA	NM_019111.3	major histocompatibility complex, class II, DR alpha
SHC1	NM_183001.4	SHC (Src homology 2 domain containing) transforming protein 1
TLR1	NM_003263.3	toll-like receptor 1
TLR2	NM_003264.3	toll-like receptor 2
TLR3	NM_003265.2	toll-like receptor 3

(Continued)

TABLE E2. Continued

Gene symbol	GeneBank Accession	Gene name
TLR4	NM_138554.2	toll-like receptor 4
TLR5	NM_003268.3	toll-like receptor 5
TLR6	NM_006068.2	toll-like receptor 6
TLR7	NM_016562.3	toll-like receptor 7
TGFBR1	NM_004612.2	transforming growth factor beta receptor 1
Signal transduction		
CREB1	NM_134442.2	cAMP responsive element binding protein 1
CLTC	NM_004859.2	clathrin, heavy chain (Hc)
CFL1	NM_005507.2	cofilin 1
CSF1	NM_000757.4	colony stimulating factor 1
C1QA	NM_015991.2	complement component 1, q subcomponent, A chain
C1QB	NM_000491.3	complement component 1, q subcomponent, B chain
C1R	NM_001733.4	complement component 1, r subcomponent
C1S	NM_001734.2	complement component 1, s subcomponent
C2	NM_000063.3	complement component 2
C3	NM_000064.2	complement component 3
C4A	NM_007293.2	complement component 4A
C5	NM_001735.2	complement component 5
C6	NM_000065.2	complement component 6
C7	NM_000587.2	complement component 7
CFB	NM_001710.5	complement factor B
FOS	NM_005252.2	FBJ murine osteosarcoma viral oncogene homolog
GUSB	NM_000181.1	glucuronidase, beta
GAPDH	NM_002046.3	glyceraldehyde-3-phosphate dehydrogenase
GNAS	NM_080425.1	GNAS complex locus
GNB1	NM_002074.2	guanine nucleotide binding protein (G protein), beta polypeptide 1
GNAQ	NM_002072.2	guanine nucleotide binding protein (G protein), q polypeptide
HSPB1	NM_001540.3	heat shock 27kDa protein 1
HSPB2	NM_001541.3	heat shock 27kDa protein 2
HPRT1	NM_000194.1	hypoxanthine phosphoribosyltransferase 1
CFD	NM_001928.2	complement factor B
FXJD2	NM_021603.3	FBJ murine osteosarcoma viral oncogene homolog
MAFK	NM_002360.3	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K
MYC	NM_002467.3	MYC associated factor X
ITGB2	NM_000211.2	integrin, beta 2
KEAP1	NM_012289.3	kelch-like ECH-associated protein 1
LIMK1	NM_002314.2	LIM domain kinase 1
MASP1	NM_139125.2	mannan-binding lectin serine peptidase 1
MKNK1	NM_003684.3	MAP kinase interacting serine/threonine kinase 1
MAPK1	NM_002745.4	mitogen-activated protein kinase 1
MAPK14	NM_001315.1	mitogen-activated protein kinase 14
MAPK3	NM_001040056.1	mitogen-activated protein kinase 3
MAPK8	NM_002750.2	mitogen-activated protein kinase 8
MAP2K1	NM_002755.2	mitogen-activated protein kinase kinase 1
MAP2K4	NM_003010.2	mitogen-activated protein kinase kinase 4
MAP2K6	NM_002758.3	mitogen-activated protein kinase kinase 6
MAP3K1	NM_005921.1	mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase
MAP3K5	NM_005923.3	mitogen-activated protein kinase kinase kinase 5
MAP3K7	NM_145333.1	mitogen-activated protein kinase kinase kinase 7
MAPKAPK2	NM_004759.3	mitogen-activated protein kinase-activated protein kinase 2
MAPKAPK5	NM_003668.2	mitogen-activated protein kinase-activated protein kinase 5
MAX	NM_145113.1	MYC-associated factor X
MYD88	NM_002468.3	myeloid differentiation primary response gene (88)
MYL2	NM_000432.3	myosin, light chain 2, regulatory, cardiac, slow
NOS2	NM_000625.4	nitric oxide synthase 2, inducible

(Continued)

TABLE E2. Continued

Gene symbol	GeneBank Accession	Gene name
NR3C1	NM_001018074.1	nuclear receptor subfamily 3, group C, member 1
PGK1	NM_000291.2	phosphoglycerate kinase 1
PLA2G4A	NM_024420.2	phospholipase A2, group IVA
PLCB1	NM_182734.1	phospholipase C, beta 1
PRKCA	NM_002737.2	protein kinase C, alpha
PRKCB	NM_212535.1	protein kinase C, beta
PPP1R12B	NM_032103.1	protein phosphatase 1, regulatory subunit 12B
PTK2	NM_005607.3	PTK2 protein tyrosine kinase 2
RAPGEF2	NM_014247.2	Rap guanine nucleotide exchange factor (GEF) 2
RHOA	NM_001664.2	ras homolog family member A
RAC1	NM_198829.1	ras-related C3 botulinum toxin substrate 1
RIPK1	NM_003804.3	receptor (TNFRSF)-interacting serine-threonine kinase 1
RIPK2	NM_003821.5	receptor-interacting serine-threonine kinase 2
ROCK2	NM_004850.3	Rho-associated, coiled-coil containing protein kinase 2
RPS6KA5	NM_004755.2	ribosomal protein S6 kinase, 90kDa, polypeptide 5
TRAF2	NM_021138.3	TNF receptor-associated factor 2
TRADD	NM_003789.2	TNFRSF1A-associated via death domain
TOLLIP	NM_019009.2	toll interacting protein
HRAS	NM_005343.2	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
RAF1	NM_002880.2	v-raf-1 murine leukemia viral oncogene homolog 1
ATF2	NM_001880.2	activating transcription factor 2
BCL6	NM_001706.2	B-cell CLL/lymphoma 6
CEBPB	NM_005194.2	CCAAT/enhancer binding protein (C/EBP), beta
DDIT3	NM_004083.4	DNA-damage-inducible transcript 3
ELK1	NM_005229.3	ELK1, member of ETS oncogene family
HMGN1	NM_004965.6	high mobility group nucleosome binding domain 1
Transcription factor		
HDAC4	NM_006037.3	histone deacetylase 4
JUN	NM_002228.3	jun proto-oncogene
MEF2A	NM_005587.2	myocyte enhancer factor 2A
MEF2C	NM_002397.3	myocyte enhancer factor 2C
MEF2D	NM_005920.2	myocyte enhancer factor 2D
NFE2L2	NM_006164.3	nuclear factor (erythroid-derived 2)-like 2
NFATC3	NM_004555.2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3
NFKB1	NM_003998.2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
STAT1	NM_007315.2	signal transducer and activator of transcription 1, 91kDa
MAFF	NM_012323.2	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F
MAFG	NM_002359.2	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G