Effect of Hydrodynamics-Based Gene Delivery of Plasmid DNA Encoding Interleukin-1 Receptor Antagonist-Ig for Treatment of Rat Autoimmune Myocarditis

Possible Mechanism for Lymphocytes and Noncardiac Cells

Hui Liu, MD; Haruo Hanawa, MD; Tsuyoshi Yoshida, MD; Raafat Elnaggar, MD; Manabu Hayashi, MD; Ritsu Watanabe, MD; Ken Toba, MD; Kaori Yoshida, BS; He Chang, MD; Yuji Okura, MD; Kiminori Kato, MD; Makoto Kodama, MD; Hiroki Maruyama, MD; Junichi Miyazaki, MD; Mikio Nakazawa, PhD; Yoshifusa Aizawa, MD

Background—Interleukin-1 (IL-1) is a powerful and important cytokine in myocarditis. The purpose of this study was to evaluate the effect and possible mechanism of hydrodynamics-based delivery of the IL-1 receptor antagonist (IL-1RA)-immunoglobulin (Ig) gene for treatment of rat experimental autoimmune myocarditis (EAM).

Methods and Results—On the day after immunization, rats were transfected with either pCAGGS encoding IL-1RA-Ig or pCAGGS encoding Ig alone. On day 17, IL-1RA-Ig gene therapy was effective in controlling EAM, as monitored by a decreased ratio of heart weight to body weight, reduced myocarditis areas, reduced gene expression of atrial natriuretic peptide in hearts, and improved cardiac function in echocardiographic and hemodynamic parameters. Examination of the expression of IL-1-related genes in purified cells from EAM hearts suggested that ectopic IL-1RA-Ig–acting target cells were αβ T cells and noncardiomyocytic noninflammatory cells such as fibroblasts, smooth muscle cells, and endothelial cells. Therefore, we examined the effect of serum containing IL-1RA-Ig on the expression of immune-relevant genes within noncardiomyocytic cells cultured from EAM hearts or concanavalin A–stimulated lymphocytes derived from lymph nodes in EAM-affected rats. The expression of immunologic molecules (prostaglandin E synthase, cyclooxygenase-2, and IL-1β) in cultivated noncardiomyocytic cells and Th1 cytokines (IL-2 and IFN-γ) in lymphocytes was significantly decreased by the serum containing IL-1RA-Ig.

Conclusions—EAM was suppressed by hydrodynamics-based delivery of plasmid DNA encoding IL-1RA-Ig. In addition, IL-1RA-Ig suppressed gene expression of prostaglandin synthases and IL-1 in noncardiomyocytic cells and Th1 cytokines in lymphocytes. (Circulation. 2005;111:1593-1600.)

Key Words: cardiomyopathy, dilated cytokines sialoglycoproteins myocarditis prostaglandins

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From the Divisions of Cardiology (H.L., H.H., T.Y., R.E., M.H., R.W., K.T., K.Y., H.C., Y.O., K.K., M.K., Y.A.) and Clinical Nephrology and Rheumatology (H.M.), Niigata University Graduate School of Medical and Dental Sciences, and Department of Medical Technology, School of Health Sciences, Faculty of Medicine (M.N.), Niigata University, Niigata, and Division of Stem Cell Regulation Research, Osaka University Medical School (J.M.), Suita, Japan.

Correspondence to H. Hanawa, Division of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata 951-8120, Japan. E-mail hanawa@med.niigata-u.ac.jp

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lular IL-1RA (icIL-1RA). IL-1RA functions as an antagonist by competitively binding to IL-1RI. IL-1RA cannot transduce intracellular signals because it is unable to bind to IL-1Racp. IL-1RA-based therapies are being evaluated for a variety of diseases.

The purpose of the present study was to investigate whether IL-1RA transduction ameliorated EAM and by what mechanisms this therapy occurred. Hydrodynamics-based gene transfer via the rapid tail vein injection of a large volume is more efficient than delivery by intramuscular injection with electroporation. This method can retrogradely deliver plasmid DNA predominantly into hepatocytes via hepatic vein. Moreover, chimeras with immunoglobulin (Ig) facilitate elevated concentration levels. In this study, we examined the efficacy of hydrodynamics-based delivery of plasmid DNA encoding an IL-1RA-Ig chimera.

**Methods**

### Animals

Seven-week-old male Lewis rats were purchased from Charles-River Laboratories, Japan (Atsugi, Kanagawa, Japan) and were maintained in our animal facilities. Throughout the studies, all the animals were treated in accordance with the guidelines for animal experiments as laid out by our institute.

### Induction of EAM

Cardiac myosin was prepared from the ventricular muscle of porcine hearts as previously described. To produce EAM, each rat was immunized on day 0 with 0.2 mL emulsion containing cardiac myosin with an equal volume of complete Freund’s adjuvant by a single subcutaneous injection in both footpads.

### In Vivo Treatment of EAM With Plasmid DNA Encoding IL-1RA-Ig Gene

We first constructed the plasmid vector pCAGGS-Ig-glucagon (Glu)-tag, containing NotI and EcoRI restriction sites, via polymerase chain reaction (PCR) amplification. For this purpose, initial PCR products were generated from rat spleen cDNA using KOD Plus DNA polymerase (Toyobo) and the following primers: 5’-gaGAATTCATTTAAATgaagaGCGGCCGCcgtgcccagaaactgtg-3’ (contains both Swol and NotI restriction sites) and 5’-tcaaccactgcaacaaatggttacacgcaggagagact-3’.

The Plasmid DNA Injection Techniques

Nineteen rats were divided into 2 groups, the pCAGGS-IL-1RA-Ig group (IL-1RA-Ig group; n = 10) and the pCAGGS-SP-Ig group (SP-Ig group; n = 9). Rats were injected with 800 μg pCAGGS-mouse IL-1RA-Ig-Glu-tag or pCAGGS-SP-Ig-Glu-tag via the tail vein within 15 seconds (receiving ~80 mL/kg body weight) on day 1.

### Plasmid Chimeric Glucagon-Glu-Tag Protein Measurement

Blood samples were taken on days 2, 5, 8, 12, and 17. Glucagon concentrations were measured with a glucagon radioimmunoassay kit (Daiichi Radioisotope Laboratories). Chimeric protein concentrations in blood were calculated with Glu-tag. To observe the relationship of them and gene expression in liver, the livers were harvested on days 2, 5, 8, 12, and 17 after injection of pCAGGS-IL-1RA-Ig-Glu-tag into normal rats (n = 4, respectively), and transgene expressions were examined by real-time reverse-transcriptase (RT) PCR using the following primers: 5’-tctgactacgccgcttccca-3’ (726 to 748 bases in pCAGGS) and 5’-atacgtatgtaacctccg-3’ (316 to 339 bases in mouse sIL-1RA).

### Evaluation of Echocardiography and Hemodynamic Parameters

On day 17, echocardiography was performed with a 7.5-MHz probe (SSD-630, Aloka ECHO camera). Left ventricular (LV) internal diameter in end diastole and end systole, interventricular septal thickness, LV posterior wall thickness, pericardial effusion (PE) under LV posterior wall thickness, and LV fractional shortening were calculated from M-mode echocardiograms over 3 consecutive cardiac cycles.

The hemodynamic parameters were measured after echocardiography. Mean arterial pressure was recorded through a catheter introduced into the right femoral artery. Central venous pressure (was recorded through a catheter introduced into the confluence of the vena cava with the right jugular vein. A catheter-tip transducer was inserted into the left ventricle from the right carotid artery to measure the peak left ventricular pressure and left ventricular end-diastolic pressure. The rates of intraventricular pressure rise and decline (+dP/dt) were measured with a differential amplifier. Heart rate was calculated from ECGs. All hemodynamic parameters were recorded on a thermostylus recorder after a stabilizing period of 10 minutes.

### Evaluation of Histopathology

Heart and body weights were measured, and the ratio of heart weight to body weight (g/g) was calculated. Several transverse sections were cut from the midventricle slice and stained with Azan-Mallory. The myocarditis area of each specimen was determined with a color image analyzer (Mac SCOPE version 2.6, Mitani Corp).

### Measurement of Atrial Natriuretic Peptide mRNA Levels

To measure mRNA levels of atrial natriuretic peptide (ANP), a heart failure marker, total RNA was isolated from the apical one third of the heart on day 17. The absolute copy number of ANP mRNA was measured by quantitative real-time RT-PCR.

### Gene Expression of IL-1 Family in EAM Hearts

To evaluate crosstalk between members of the IL-1 family, the mRNA levels of IL-1α, IL-1β, IL-1RI, IL-1RII, IL-1Racp, sIL-1RA, or total IL-1RA (sIL-1RA + icIL-1RA) in both isolated and purified cells from EAM hearts were measured. On day 18, cardiomyocytes and the other cells in the hearts of EAM rats were isolated after collagenase perfusion treatment for 20 minutes with a Langendorff apparatus as reported previously. Isolated cells, while maintained in an isotonic buffer, were separated serially through stainless steel sieves into cardiomyocytes and the other cells. Because the inflammatory cells are almost CD11b+ cells (macrophages/dendritic cells/granulocytes) and αβT cells, the other cells without cardiomyocytes were separated into αβT cells, CD11b+ cells, and noncardiomyocyte noninflammatory (NCNI) cells (mainly fibroblasts, smooth
TABLE 1. Absolute Copy Numbers of Specific Cell Marker mRNA in Cultivated Cells

<table>
<thead>
<tr>
<th></th>
<th>NC Cells (n=6)</th>
<th>Lymph Node Cells (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>37 600±6300</td>
<td>1 490 000±215 000</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>149 000 000±10 500 000</td>
<td>170 000±15 000</td>
</tr>
<tr>
<td>Calponin</td>
<td>23 400 000±2 110 000</td>
<td>ND</td>
</tr>
<tr>
<td>CD11b</td>
<td>11 800 000±977 000</td>
<td>19 000±4200</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>194 000±31 000</td>
<td>262 000±56 000</td>
</tr>
<tr>
<td>α-Cardiac myosin</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM.

muscle cells, and endothelial cells) by anti-PE micro beads (Miltenyi Biotech) and an MACS magnetic cell sorting system (Miltenyi Biotech) using appropriate monoclonal antibodies, namely PE-conjugated TCR/β (R73) and CD11b (OX-42) (Pharmingen). The fractions of cardiomocytes, αβT cells, CD11b+ cells, and NCNI cells were confirmed by analysis of specific marker gene expression—a cardiac myosin, CD3, CD11b, collagen type III, calponin, and von Willebrand factor—and even if the level of contamination was the highest, it was <10% (data not shown). Total RNA was isolated from each purified cell fraction (cardiomycocytes, n=5; αβT cells, n=5; CD11b+ cells, n=5; NCNI cells, n=6). The absolute copy numbers of IL-1 family mRNA were measured by quantitative real-time RT-PCR.

Cell Culture With Serum Containing IL-1RA-Ig

**NC Cells**

On day 18, NC cells were isolated from the hearts of EAM rats via collagenase preparation and were cultured for 1 week on 35-mm well dishes in 3 mL RPMI medium supplemented with 10% FCS. These cultivated NC cells were suggested to contain mainly fibroblasts, smooth muscle cells, and CD11b+ cells, as determined by gene expression analysis (Table 1). After reaching confluence, NC cells were stimulated by addition of 10 ng/mL IL-1α (Pepro Tech) and 100 μL IL-1RA-Ig-Glu-tag—containing serum (30 nmol/L) or the same amount of Ig-Glu tag—containing serum (IL-1RA-Ig+IL-1α group, n=6; SP-Ig+IL-1α group, n=6; no serum and no IL-1α group, n=6). After culture for 24 hours at 37°C, NC cells were collected and total RNA was isolated. The absolute copy numbers of γ-actin, prostaglandin E synthase (PGES), cyclooxygenase-2 (Cox-2), and IL-1β mRNA were measured by quantitative real-time RT-PCR.

**Lymphocytes**

Lymphocytes isolated from popliteal lymph nodes of EAM rat were prepared in 3 mL RPMI medium supplemented with 10% FCS in 35-mm–well dishes. These cells were thought to be mainly lymphocytes, as determined by expression of the CD3 gene (Table 1). Because transfer of concanavalin A (Con-A)–stimulated lymphocytes from EAM popliteal lymph nodes could induce EAM27 and Con-A–stimulated lymphocytes can express the IL-1RI gene (data not shown), we stimulated these cells with 10 ng/mL Con-A (Sigma) and 10 ng/mL IL-1α at 6×10^6 cells per dish. One hundred microliters of IL-1RA-Ig-Glu-tag—containing serum (30 nmol/L) or the same amount of Ig-Glu tag—containing serum was added (IL-1RA-Ig+Con-A+IL-1α, n=6; SP-Ig+Con-A+IL-1α, n=6; no serum, no Con-A, and no IL-1α, n=6). After culture for 24 hours at 37°C, these cells were collected and total RNA was isolated. The absolute copy numbers of γ-actin, IL-2, and IFN-γ mRNA were measured by quantitative real-time RT-PCR.

**Quantitative Real-Time RT-PCR Analysis**

Total RNA was extracted by use of Trizol (Invitrogen). cDNA was synthesized from 2 to 5 μg total RNA with random primers and murine Moloney leukemia virus RT. To create the plasmids used for the standard, the cDNAs for ANP, specific cell markers, IL-1 family proteins, and immunologic molecules were amplified from an EAM heart–derived cDNA library with the primers indicated in Table 2. PCR-amplified cDNA inserts were directly inserted into the pGEM-T easy vector, and the recombinant plasmids were isolated, after transformation into E coli JM109 competent cells, with the MagExtractor plasmid kit (Toyobo). Diluted plasmid and cDNA were amplified via real-time RT-PCR with a Lightcycler, together

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**TABLE 2. List of Primers for Quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Sense Primer</th>
<th>Antisense Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP</td>
<td>5'-gttcaccatctctgctaatccac-3'</td>
</tr>
<tr>
<td>α-Cardiac myosin</td>
<td>5'-agaagaaaagatagacatggtgcc-3'</td>
</tr>
<tr>
<td>CD3</td>
<td>5'-gtgatggagaacagccaggt-3'</td>
</tr>
<tr>
<td>CD11b</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>Calponin</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>IL-1α</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>IL-1RI</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>IL-1RII</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>IL-1Rap</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>sIL-1RA</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>Total IL-1RA</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>IL-2</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>PGES</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>Cox-2</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
<tr>
<td>γ-Actin</td>
<td>5'-tgtgatattctcaaacaggagcat-3'</td>
</tr>
</tbody>
</table>
with the same primer pair used for making the plasmid and a LightCycler-FastStart DNA Master SYBR Green I kit (Roche). After an initial denaturation step of 10 minutes at 95°C, a 3-step cycling procedure (denaturation at 95°C for 10 seconds, annealing at 62°C for 10 seconds, and extension at 72°C for 13 seconds) was used for 40 cycles. The absolute copy numbers of particular transcripts were calculated by LightCycler software using a standard curve approach. cDNA from cultivated cells was then subjected to quantitative RT-PCR, and concentration of IL-1RA-Ig-Glu-tag and Ig-Glu-tag were expressed as mean ± SEM.

Statistical Analysis
Statistical assessment was performed by unpaired Student t test or 1-way ANOVA and Bonferroni multiple comparison test. The differences were considered significant at P<0.05. Ratio of heart weight to body weight, myocarditis area, echocardiography and hemodynamic parameters, data obtained from quantitative RT-PCR, and concentration of IL-1RA-Ig-Glu-tag and Ig-Glu-tag were expressed as mean ± SEM.

Results
Effect of In Vivo Treatment With Plasmid DNA Encoding IL-1RA-Ig Gene
The heart to body weight ratios in the IL-1RA-Ig group were significantly lower than those of the SP-Ig group (mean ± SEM, 0.53 ± 0.03% versus 0.59 ± 0.05%; P = 0.0043) (Figure 1A). The inflammatory area in the IL-1RA-Ig group was significantly smaller than that observed in the SP-Ig group (22.3 ± 4.8% versus 34.6 ± 6.8%, P = 0.0003) (Figure 1B). Expression of ANP mRNA (a heart failure marker) was significantly lower in heart tissues of the IL-1RA-Ig group than those of controls (7.13 × 10⁶ ± 3.62 × 10⁶ versus 31.6 × 10⁶ ± 24.2 × 10⁶ copy/total RNA μg; P = 0.0084) (Figure 1C).

Echocardiographic and Hemodynamic Parameters
As shown in Table 3, the LV fractional shortening and the absolute value of +dP/dt or −dP/dt in IL-1RA-Ig group were significantly larger than in SP-Ig group. LV end-systolic pressure, and central venous pressure were significantly smaller in the IL-1RA-Ig group than in SP-Ig group.

Time Course of IL-1RA-Ig-Glu-Tag Protein Levels
Plasma IL-1RA-Ig-Glu-tag protein levels in the IL-1RA-Ig group were found to increase, peaking at 23.21 ± 8.52 nmol/L (mean ± SEM) on day 2, and gradually decrease to 5.56 ± 2.70 nmol/L on day 5, 1.64 ± 0.63 nmol/L on day 8, 0.85 ± 0.45 nmol/L on day 12, and 0.22 ± 0.07 nmol/L on day 17. The plasma Ig-Glu-tag protein levels in the SP-Ig group were seen lower.

Table 3. Echocardiographic and Hemodynamic Parameters

<table>
<thead>
<tr>
<th></th>
<th>IL-1RA-Ig</th>
<th>SP-Ig</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=7)</td>
<td>(n=6)</td>
<td></td>
</tr>
<tr>
<td>LVEDd, mm</td>
<td>5.41 ± 0.05</td>
<td>5.48 ± 0.29</td>
<td>0.81</td>
</tr>
<tr>
<td>LVESd, mm</td>
<td>3.01 ± 0.17</td>
<td>3.81 ± 0.24</td>
<td>0.018</td>
</tr>
<tr>
<td>IVS, mm</td>
<td>1.84 ± 0.05</td>
<td>1.96 ± 0.12</td>
<td>0.33</td>
</tr>
<tr>
<td>LVPW, mm</td>
<td>1.89 ± 0.17</td>
<td>2.24 ± 0.27</td>
<td>0.012</td>
</tr>
<tr>
<td>PE, mm</td>
<td>1.71 ± 0.47</td>
<td>3.67 ± 0.49</td>
<td>0.016</td>
</tr>
<tr>
<td>LVFS, %</td>
<td>44.4 ± 2.9</td>
<td>30.4 ± 2.4</td>
<td>0.0039</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>354.9 ± 14.3</td>
<td>381.5 ± 19.8</td>
<td>0.29</td>
</tr>
<tr>
<td>CVP, mm Hg</td>
<td>1.73 ± 0.26</td>
<td>3.93 ± 0.55</td>
<td>0.0029</td>
</tr>
<tr>
<td>AP, mm Hg</td>
<td>78.4 ± 2.52</td>
<td>72.6 ± 1.72</td>
<td>0.094</td>
</tr>
<tr>
<td>LVP, mm Hg</td>
<td>91.1 ± 3.53</td>
<td>85.9 ± 2.08</td>
<td>0.244</td>
</tr>
<tr>
<td>EDP, mm Hg</td>
<td>11.7 ± 1.3</td>
<td>18.4 ± 2.24</td>
<td>0.0218</td>
</tr>
<tr>
<td>+dP/dt, mm Hg/s</td>
<td>4459 ± 243</td>
<td>3636 ± 178</td>
<td>0.0226</td>
</tr>
<tr>
<td>−dP/dt, mm Hg/s</td>
<td>−5547 ± 352</td>
<td>−4129 ± 202</td>
<td>0.0067</td>
</tr>
</tbody>
</table>

LVEDd indicates LV end-diastolic internal diameter; LVESd, LV end-systolic internal diameter; IVS, interventricular septal thickness; LVPW, LV posterior wall thickness; LVFS, LV fractional shortening; HR, heart rate; CVP, central venous pressure; AP, mean blood pressure; LVP, peak LV pressure; EDP, LV end-diastolic pressure; +dP/dt, maximum dP/dt; and −dP/dt, minimum dP/dt. Result are expressed as mean ± SEM.
to increase, peaking at 22.75±2.34 nmol/L on day 2, and gradually decrease to 19.48±1.62 nmol/L on day 5, 17.50±2.50 nmol/L on day 8, 15.59±5.56 nmol/L on day 12, and 7.45±1.88 nmol/L on day 17 (Figure 2A). The expressions of IL-1RA-Ig-Glu-tag transferred into normal rat liver by hydrodynamics-based gene delivery were similar to plasma IL-1RA-Ig-Glu-tag protein levels (Figure 2B). It has been reported that IL-1RA (1 to 10 ng/mL, 0.05 to 0.5 nmol/L) suppresses the production of PGES from rat astrocytes stimulated by lipopolysaccharide in vitro.28 These results indicated that continuous effective delivery of IL-1RA-Ig protein for 16 days can be achieved in rats by hydrodynamics-based transfection.

Gene Expression of IL-1 Family in Purified Cells From EAM Hearts

Both IL-1α and IL-1β were strongly expressed in CD11b+ cells. The IL-1RI gene was strongly expressed in NCNI cells (the cell fraction containing mainly fibroblasts, smooth muscle cells, and endothelial cells) and αβT cells, whereas the IL-1RII gene was found to be marked expressed in CD11b+ cells. The IL-1Racp gene was strongly expressed in both CD11b+ and NCNI cells and was moderately expressed in αβT cells. Both total IL-1RA and sIL-1RA were detected in CD11b+ cells. These results suggested that IL-1, produced mainly by CD11b+ cells, acted on NCNI and αβT cells by binding to IL-1RI and transduced intracellular signals by forming with IL-1Racp. On the other hand, CD11b+ cells also produced native IL-1RA and IL-1RII, potentially suppressing the action of IL-1 (Figure 3).

Expression of Immunologic Molecules in Cultivated Cells With Serum Containing IL-1RA-Ig

IL-1RA-Ig-containing serum significantly reduced expression of PGES (mean±SEM, 1.22±0.22 versus 1.94±0.17; P<0.0001), Cox-2 (0.93±0.24 versus 2.00±1.05; P=0.0092), and IL-1β (3.91±1.22 versus 11.25±2.16; P<0.0001) at the mRNA level in cultivated NC cells (the cell fraction containing mainly fibroblasts, smooth muscle cells, and CD11b+ cells) (Figure 4). In addition, IL-1RA-Ig-containing serum significantly reduced expression of the IL-2 (0.0004±0.0001 versus 0.0310±0.0090; P<0.0001) and IFN-γ (0.0065±0.0036 versus 0.0461±0.0385; P=0.0091) genes in cultivated lymphocytes (Figure 5).

Discussion
In the present study, we demonstrated that hydrodynamics-based delivery of plasmid DNA encoding the IL-1RA-Ig gene ameliorated EAM. IL-1RA-Ig-affecting cells were thought to be NCNI cells (fibroblasts, smooth muscle cells, and endothelial cells) and αβT cells because IL-1RI and IL-1Racp were found in them. Endogenous IL-1α and IL-1β produced mainly by CD11b+ cells, especially secreted IL-1β, influence these surrounding cells in a paracrine manner. IL-1RA-Ig, generated after gene transfer, appears to inhibit the IL-1–induced reactions of these cells and ameliorates EAM.

The functions of IL-1 with respect to cell regulation are varied.29 In this study, the effect of serum containing IL-1RA-Ig on the mRNA expression of various immunologic molecules in cultivated NC cells (mainly fibroblasts, smooth muscle cells, and CD11b+ cells) from hearts and lymphocytes from popliteal lymph nodes of EAM rats was investigated. The concentration of IL-1RA-Ig in cultivated cells was almost the same as that observed at the onset of myocarditis (day 10 to 11) and that reported by previous in vitro studies to be an effective dose.28,30 In this study, there was a 30- to 50-fold increase in the expression of PGES, Cox-2, and IL-1β mRNA in NC cells by IL-1 alone, but the increase was significantly reduced by serum containing IL-1RA-Ig. PGE2 produced by PGES plays an important role in inflammation and pain.31 In rheumatoid arthritis, the level of PGES detected

Figures 2A and 2B show the plasma IL-1RA-Glu-tag protein and Ig-Glu-tag protein levels, and the absolute copy numbers of IL-1RA mRNA in liver. The error bars represent SEM. IL-1RA-Ig-Glu-tag indicates rats injected with pCAGGS-mouse IL-1RA-Ig-Glu-tag; Ig-Glu-tag, rats injected with pCAGGS-rat SP-Ig-Glu-tag.
at the inflammatory region is very high. \(^3^2\) Our study of PGES and Cox-2 gene expression in purified cells from EAM hearts indicated that they were produced mainly by NCNI cells and slightly by CD11b\(^+\) cells (data not shown). IL-1RA-Ig may inhibit the expression of the PGES and Cox-2 gene directly on NCNI cells via IL-1R. IL-1–induced IL-1 production has been shown in various cell types. \(^3^3\) IL-1RA-Ig may also inhibit IL-1 production directly on NCNI cells. However, because our study indicated that IL-1 was produced mainly by CD11b\(^+\) cells, IL-1RA-Ig may inhibit IL-1 production of CD11b\(^+\) cells indirectly via NCNI cells or \(\alpha\beta\)T cells. Reduced PGES, Cox-2, and/or IL-1 production by NC cells in EAM hearts may be an effect resulting in improved myocarditis. On the other hand, in lymphocytes of popliteal lymph node, IL-2 and IFN-\(\gamma\) mRNA expression levels were significantly reduced by serum containing IL-1RA-Ig. In EAM,

**Figure 3.** A through G, Absolute copy numbers of IL-1\(\alpha\), IL-1\(\beta\), IL-1RI, IL-1RII, IL-1Racp, sIL-1RA, and total IL-1RA, respectively. Each cell was separated and purified from EAM heart on day 18. Error bars represent SEM. Statistical assessment was performed by 1-way ANOVA and Bonferroni multiple comparison test. Differences were considered significant at \(P<0.05\).

**Figure 4.** Copy numbers of various immunologic molecules mRNA/copy numbers of \(\gamma\)-actin mRNA in NC cells cultivated from EAM hearts. A, PGES; B, Cox-2; C, IL-1\(\beta\). Negative control cells were cultivated in medium without IL-1\(\alpha\) and rat serum. SP-Ig + IL-1 cells were cultivated in medium with IL-1\(\alpha\) and rat serum treated with pCAGGS-SP-Ig-Glu-tag; IL-1RA-Ig + IL-1 cells were cultivated in medium with IL-1\(\alpha\) and rat serum treated with pCAGGS-mouse IL-1RA-Ig-Glu-tag. Error bars represent SEM. Statistical assessment was performed by 1-way ANOVA and Bonferroni multiple comparison test. Differences were considered significant at \(P<0.05\).
which is a T cell–mediated disease. Th1 cytokines such as IL-2 and IFN-γ produced by CD4+ T cells are thought to play a central role. Therefore, inhibition of Th1 cytokine production by IL-1RA-Ig may improve EAM.

In human rheumatoid arthritis, it has been reported that Anakinra (a recombinant form of IL-1RA) suppressed progression of this disease, and treatment with IL-1RA has been investigated in various animal models. Therefore, the number of diseases in which IL-1RA has a therapeutic relevance may be extended in the near future. Human myocarditis is serious and often fatal despite intensive care. However, the cause of myocarditis is not usually evident. It was reported that gene therapy with IL-1RA expression plasmid was effective in the treatment of viral myocarditis and reduced virus titer in hearts. Here, we demonstrated that plasmid was effective in the treatment of viral myocarditis and IL-1RA-Ig gene transfer ameliorated EAM resembling human giant cell myocarditis. Therefore, treatment with IL-1RA may be effective for acute and fulminant myocarditis even if its cause is unknown.

Hydrodynamics-based gene delivery of plasmid DNA as used in this study is both inexpensive and highly effective in terms of facilitating gene expression. The concentration of IL-1RA-Ig in blood obtained by this method was higher compared with plasmid DNA transfection into muscle with in vivo electroporation. The producing cells to which plasmid DNA is transferred by this method are thought to be mainly hepatocytes. Plasmid DNA is thought to be delivered into hepatocytes by retrograde blood flow from hepatic veins. If plasmid DNA encoding IL-1RA-Ig can be directly transfected into the heart, T cells and NCNI cells in EAM hearts will be highly influenced by IL-1RA-Ig, and EAM may be ameliorated. Hou et al reported heart-targeted plasmid DNA transfer by retrograde coronary vein using a balloon catheter.

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Effect of Hydrodynamics-Based Gene Delivery of Plasmid DNA Encoding Interleukin-1 Receptor Antagonist-Ig for Treatment of Rat Autoimmune Myocarditis: Possible Mechanism for Lymphocytes and Noncardiac Cells
Hui Liu, Haruo Hanawa, Tsuyoshi Yoshida, Raafat Elnaggar, Manabu Hayashi, Ritsuo Watanabe, Ken Toba, Kaori Yoshida, He Chang, Yuji Okura, Kiminori Kato, Makoto Kodama, Hiroki Maruyama, Junichi Miyazaki, Mikio Nakazawa and Yoshifusa Aizawa

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