Local Expression of Interleukin-1 Receptor Antagonist by Plasmid DNA Improves Mortality and Decreases Myocardial Inflammation in Experimental Coxsackieviral Myocarditis

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Background—The inflammatory cytokines have an important role in the pathogenesis of viral myocarditis. Interleukin-1 (IL-1) is one of the major cytokines that modulate the outcome of viral infection. Among the methods for in vivo gene transfer, direct injection of plasmid DNA is one that is simple and feasible. In this study, we expressed human IL-1 receptor antagonist (hIL-1Ra) in the mouse heart by direct injection of a novel plasmid vector and evaluated its effects on coxsackieviral (CVB3) myocarditis.

Methods and Results—A plasmid vector expressing hIL-1Ra (total 40 μg/mouse) was injected into the heart apex of 8-week-old inbred female Balb/C mice (day 3). On day 0, mice (IL-1Ra-CVB3, n=35) were infected intraperitoneally with 10⁴ PFU of CVB3; control mice (pCK-CVB3, n=15) were injected with empty vector on day 3 and injected on day 0. hIL-1Ra was expressed in the heart, reached its peak on day 5, and persisted for 2 weeks. The 14-day survival rate of IL-1Ra-CVB3 was higher (77%) than that of controls (30%, P<0.01). Myocardial virus titers on day 3 were lower in IL-1Ra-CVB3 mice. Myocardial inflammation on day 7 and fibrosis on day 14 were marked decreased in IL-1Ra-CVB3.

Conclusion—These results showed that direct injection of the expression plasmid vector into the heart was an effective method to transfer the cytokine gene in vivo, and expressed IL-1Ra in the heart can modulate the deleterious effect of the host immune response in viral myocarditis. (Circulation. 2002;105:1278-1281.)

Key Words: myocarditis • interleukin • gene therapy

Among the etiologic viruses of viral myocarditis, enteroviruses, in particular coxsackievirus B, are the most common.1–4 In the pathogenesis of viral myocarditis, both direct viral injury and the immune response of the host play an important role.5,6 The results from experimental viral myocarditis indicate that the immune response not only plays an important protective role, but also has deleterious effects on the host.6 The balance between these protective and deleterious effects may ultimately determine the course of viral heart disease. Inflammatory cytokine mRNAs were induced and persisted up to 80 days in experimental viral myocarditis, and the levels of interleukin-1β (IL-1) have a correlation with myocardial fibrosis.7

IL-1 receptor antagonist (IL-1Ra) binds to the IL-1 receptor and competitively inhibits the local inflammatory effects of IL-1.8 Infusion of IL-1Ra during the short phase of experimental myocarditis could decrease myocardial inflammation and mortality.9,10 These findings suggest that anticytokine therapy may suppress the deleterious effect of the host immune response. Local expression of IL-1Ra may have more therapeutic effect on viral myocarditis than systemic infusion because of its short half-life in serum.

In the present study, we expressed human IL-1Ra in the mouse heart by direct injection of a novel and highly effective mammalian expression plasmid vector (pCK) and evaluated the effects of overexpressed hIL-1Ra on CVB3 myocarditis.

Plasmid DNA

cDNA encoding hIL-1Ra was cloned by reverse transcription-polymerase chain reaction from total RNA prepared from human peripheral blood lymphocytes. The primer sequences used were 5' AAGCTTGTAGCAGCTCAGGAGGCCTCCGCAGTCAC-3' and 5' GTCGACCTACTCGTCCTCCTCGGAAGTGAAAGTTTGGTG-3'. The amplified cDNA was initially cloned into a pGEM-72f(+) plasmid (Promega). Following sequence confirmation, the hIL-1Ra cDNA was cloned into the mammalian expression vector (pCK), resulting in a pCK-IL-1Ra construct.11 pCK-IL-1Ra plasmid was purified using the EndoFree plasmid Maxi prep kit (Qiagen), diluted to 1 μg/μL in PBS (pH 7.4), and stored at −20°C.

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Intracardiac Injection of Plasmid cDNA
All procedures were reviewed and approved by the Animal Care and Use Committee at SoonChunHyang University Hospital, Bucheon, Korea. Eight-week-old inbred female Balb/C mice (maintained at Korean Research Institute of Chemical Technology; original strains from Harlan, Indianapolis, Ind) were anesthetized by intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (5 mg/kg) mixture. After a vertical neck incision and blunt dissection, a tracheostomy was performed. The mice were mechanically ventilated on an animal ventilator (CWE Inc) at a tidal volume of 0.5 cm³/stroke and a respiratory rate of 100 beats/min. Left thoracotomy was performed at the fifth intercostal space, and the apex of the heart was injected with 40 µg of pCK-IL-1Ra cDNA in 40 µL PBS using an insulin syringe. Successful injection into the myocardium produced an obvious blanching in the apex. The skin at the neck and chest was closed. Mice were extubated when spontaneous movement was observed. Immediate postoperative mortality was less than 5%, and the primary result was of pneumothorax. The day of plasmid vector injection was defined as day −3.

Murine Viral Myocarditis
CVB3 was derived from the infectious cDNA copy of the cardiotropic H3 strain of CVB3. Both the control group (pCK-CVB3, n=15) and pCK-IL-1Ra-injected group (IL-1Ra-CVB3, n=35) were infected on day 0 by intraperitoneal injection with 10⁷ PFU of CVB3. Mice were euthanized, and the serum and hearts were collected at days 3, 7, and 14. The euthanized mice (n=5, each day) were excluded from the survival study.

Organ Virus Titer and Histological Analysis
The base parts of the hearts were homogenized in Dulbecco’s Modified Eagle’s Medium containing 2% fetal calf serum. After centrifugation, the viral titers in the supernatant were determined by the plaque-forming assay. The apical parts of the hearts were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin and Masson’s trichrome. Sections were analyzed using a score of 0 represented no myocarditis and 4 represented widespread confluent inflammation.

Enzyme-Linked Immunosorbent Assay (ELISA) for hIL-1Ra Levels
The levels of hIL-1Ra were measured using an ELISA kit (R&D Systems). Briefly, the apical parts of the hearts were homogenized in lysis buffer (25 mmol/L Tris-HCl pH 7.4, 50 mmol/L NaCl, 0.5% Na-deoxycholate, 2% NP-40, 0.2% sodium dodecyl sulfate, and protease inhibitors). After centrifugation, the supernatants were used to measure the level of hIL-1Ra (pg/mL) and were normalized to the amount (mg) of total protein extracted from heart. The sera were directly used for assays. Serial dilutions of recombinant murine IL-1Ra were used as a standard.

Statistics
Data are presented as mean±SEM. Survival was analyzed using the Kaplan-Meier method. The Student’s t test was used for the analysis of numeric parameters (SPSS 10.0 for Windows, SPSS Inc). Differences were considered significant at P<0.05.

Results
Time Course of hIL-1Ra Expression
The levels of heart hIL-1Ra on day 3 (2160±1651 pg/mL/mg of total protein), day 5 (2234±713), day 7 (695±394), day 9 (1062±383), and day 14 (1221±456) were significantly higher than for controls (24±3). The levels of hIL-1Ra in the heart peaked on day 5 and lasted for 2 weeks (Figure 1A). In more than 70% of pCK-IL-1Ra injected hearts with CVB3 infection, hIL-1Ra was expressed at levels at least 3-fold higher than in control hearts (data not shown). Serum hIL-1Ra was not significantly elevated.

Survival Rates
In the 10 pCK-IL-1Ra-injected mice without CVB3 infection, no mice died during the 4 weeks after the operation. The pCK-IL-1Ra-injected mice with CVB3 infection (IL-1Ra-CVB3, n=35) showed a significantly higher survival (77%) than did the pCK-CVB3 group (n=15, 30%, P<0.01) at day 14. Four weeks after the infection, the survival rate of IL-1Ra-CVB3 was significantly higher than in pCK-CVB3 (48% versus 10%, P<0.01; Figure 1B). These data suggest that expressed hIL-1Ra had a therapeutic effect on the mortality of viral myocarditis.
In viral myocarditis, virus proliferation in myocytes can induce direct cytotoxicity, independent of an immune response, and some coxsackieviruses can cause direct myocyte damage.13 The host immune responses may induce tissue damage by the protective response to remove virus-infected myocytes, or by inappropriate cardiac injury, where the heart is attacked mostly by sensitized T-lymphocytes.14 This immune response should be specific, attacking only infected cells; however, an imbalance in the immune response may lead to either an overwhelming virus-induced myocardial injury or predominantly immune-mediated tissue damage.6

IL-1Ra inhibits many actions of IL-1 by competing for its receptor. The balance between endogenous IL-1 and IL-1Ra in vivo is an important determinant of the host response to infection.8 In the murine myocarditis model, the increased levels of IL-1β correlated with the myocardial fibrosis,7 whereas the increased serum levels of IL-1Ra, which was delivered by electroporation with the plasmid vector or continuous IL-1Ra infusion, improved the survival rates and decreased myocardial inflammation and fibrosis.9,10 These findings suggest that anticytokine therapy may modulate the deleterious effect of the host immune response in viral myocarditis.

IL-1β has negative inotropic effects and cytotoxicity through excessive NO production by inducible nitric oxide synthase in viral myocarditis.10,15 IL-1β may also activate fibroblasts, which affect the remodeling process after myocardial injury.16 To inhibit the IL-1 response in the cells that express IL-1 receptor, a 10- to 100-fold excess of IL-1Ra is required.17 In this study, we could overexpress the IL-1Ra in the hearts 100-fold higher than in control hearts by direct injection with a highly effective and novel plasmid vector. The local expression of IL-1Ra suppressed viral replication, myocardial inflammation, and subsequent fibrosis in the heart, and improved the survival rates.

In conclusion, we demonstrated that direct injection of the expression plasmid vector into the heart was an effective method to transfer the cytokine gene in vivo and anticytokine therapy can modulate the deleterious effect of the host immune response in viral myocarditis.

**References**


**Discussion**

In this study, we were able to overexpress hIL-1Ra in the heart by using a direct injection of a plasmid vector (pCK-IL-1Ra) and show the therapeutic effect of hIL-1Ra on murine CVB3 myocarditis. The overexpressed hIL-1Ra in the hearts improved survival rates, decreased the myocardial inflammation, inhibited viral proliferation in the heart, and prevented subsequent fibrosis.

**Changes of Viral Titers and Histological Findings**

In IL-1Ra-CVB3 mice, the viral titers in the hearts were significantly lower than in pCK-CVB3 mice at days 3 (5.13±0.19 versus 6.21±0.16 log10 PFU/mg heart; P=0.002, n=5 in each group). In day 7 hearts, the viral titers were decreased in both groups and showed no differences (1.15±0.30 versus 2.29±0.23, P>0.05; Figure 1C). The myocardial inflammation on days 7 (3.0±0.5) and 14 (2.0±0.5) was markedly decreased in IL-1Ra-CVB3 when compared with the pCK-CVB3 group (2.0±0.5 at day 7, 1.5±0.5 at day 14, P<0.05; Figure 2A, 2B, 2D, and 2E). The fibrosis on day 14 was also decreased in the IL-1Ra-CVB3 group (Figure 2C and 2F).

**Figure 2.** Effects of expressed hIL-1Ra on viral myocarditis and fibrosis. Myocardial inflammation on day 7 (A, D) and day 14 (B, E), and fibrosis and ventricular dilatation (C, F) on day 14 was markedly decreased in the hIL-1Ra expressed group (D through F) when compared with the infected control group (A through C). A through D, hematoxylin-and-eosin staining, magnification ×100; E and F, Masson’s Trichrome staining, magnification ×12.

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