Infusion of adrenomedullin improves acute myocarditis via attenuation of myocardial inflammation and edema

Bobby Yanagawa¹, Masaharu Kataoka¹, Shunsuke Ohnishi¹,⁎,¹, Makoto Kodamaᵇ, Koichi Tanakaᵃ, Yoshinori Miyaharaᵃ, Hatsue Ishibashi-Uedaᶜ, Yoshifusa Aizawaᵇ, Kenji Kangawaᵈ, Noritoshi Nagayaᵃ,⁎

¹ Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan
ᵇ Division of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
ᶜ Department of Pathology, National Cardiovascular Center, Osaka, Japan
ᵈ Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan

Received 23 January 2006; received in revised form 7 May 2007; accepted 24 May 2007
Time for primary review 23 days

Abstract

Objective: Our aim was to assess whether adrenomedullin (AM), a potent vasodilator peptide with a variety of cardioprotective effects, has a therapeutic potential for the treatment of acute myocarditis in a rat model.

Methods: One week after myosin injection, rats received a continuous infusion of AM or vehicle for 2 weeks, and pathological and physiological investigations were performed.

Results: AM treatment significantly reduced the infiltration of inflammatory cells in myocarditic hearts, and decreased the expressions of macrophage chemotactic protein-1, matrix metalloproteinase-2 and transforming growth factor-β. Myocardial edema indicated by increased heart weight to body weight ratio and wall thickness was attenuated by AM infusion (5.7±0.5 vs. 6.5±0.4 g/kg, and 1.9±0.3 vs. 2.8±0.5 mm, respectively). Infusion of AM significantly improved left ventricular maximum dP/dt and fractional shortening of myocarditic hearts (4203±640 vs. 3450±607 mm Hg/s, and 21.3±4.1 vs. 14.7±5.1%, respectively).

Conclusion: Infusion of AM improved cardiac function and pathological findings in a rat model of acute myocarditis. Thus, infusion of AM may be a potent therapeutic strategy for acute myocarditis.

© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Autoimmune myocarditis; Adrenomedullin; Angiogenesis; Inflammation

1. Introduction

Acute myocarditis is a non-ischemic heart disease characterized by myocardial inflammation and edema. This disease is associated with rapidly progressive heart failure, arrhythmias and sudden death [1]. Although early evidence...
of apoptosis [8], induction of angiogenesis [9] and attenuation of myocardial hypertrophy [10]. Interestingly, AM has also been shown to decrease endothelial hyperpermeability in the heart [11]. These findings raise the possibility that infusion of AM may attenuate myocardial inflammation and edema in acute myocarditis. Although previous findings have demonstrated that infusion of AM is effective for heart failure, its therapeutic effects in acute myocarditis are still unknown.

Experimental autoimmune myocarditis can be induced in rats by immunizing them with cardiac myosin, providing a model that resembles human giant cell myocarditis [12,13]. Although the majority of acute myocarditis is linked to a viral infection such as coxsackievirus B3, this viral infection can in some cases cause an autoimmune myocarditis with chronic myocardial inflammation without viral persistence, due to the exposure of autoantigens such as cardiac myosin to the immune system [14,15].

Thus, the purposes of this study were 1) to investigate whether infusion of AM improves cardiac function and pathological findings including myocardial inflammation and edema in rats with myosin-induced myocarditis, and 2) to investigate the underlying mechanisms responsible for the effects of AM.

2. Methods

2.1. Experimental autoimmune myocarditis

Purified cardiac myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [16]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl mixed with an equal volume of complete Freund’s adjuvant (CFA) containing 11 mg/ml of Mycobacterium tuberculosis (Difco Laboratories, Sparks, MD, USA).

Male 10-week-old Lewis rats were used in the present study. Rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg) and were given an injection of either 0.2 ml of antigen–adjuvant emulsion or saline mixed with CFA into the footpad. One week after myosin injection, an osmotic pump (Alzet, Cupertino, CA, USA) was filled with either AM (0.05 μg/kg/min) or PBS for 2 weeks, and implanted subcutaneously between the scapulae. This protocol resulted in the creation of 3 groups (n = 11 in each group): sham rats given PBS (sham group), myosin-treated rats given PBS (control group), and myosin-treated rats given AM (AM group). The dose of AM used in this study has anti-apoptotic effects without significant hypotension [8]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. Histopathology

After completion of hemodynamic measurements on day 21 post-myosin injection, the heart was excised above the origin of the great vessels, and ventricular weight was recorded. Midventricular portions of the heart were formalin-fixed and embedded in paraffin, and 4 μm-sections were cut and stained with hematoxylin and eosin (H&E). H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) as described previously [17]. Briefly, coagulation necrosis, granulation, inflammation and edema were evaluated without knowledge of the experimental groups on the following scale: 0, no or questionable presence; 1, limited focal distribution less than 25% area of the section; 2, intermediate severity covering less than 50% area of the section; 3, intermediate severity covering greater than 50% and less than 75% area of the section; and 4, coalescent and extensive foci more than 75% area to the entirety of the transversely sectioned ventricular tissue (5 fields per rat, n = 8 in each group).

2.3. Picrosirius red staining

Paraffin-embedded sections were submitted for picrosirius staining for total collagen distribution. Slides were hydrated, placed in Weigert’s iron hematoxylin and in Bouin’s fluid (70% saturated aqueous picric acid, 5% acetic acid, 25% formalin) for 10 min. The slides were rinsed in distilled water and placed in 0.025% picrosirius red solution overnight. The sections were rinsed, dehydrated, cleared, and mounted. Amount of collagen stain was quantitated using image analysis software on high-powered (×200) cross-sectional images (10 fields per rat, n = 5 in each group).

2.4. Immunohistochemistry

Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Immunohistochemical staining of the sections was performed with antibodies raised against macrophage chemoattractant protein-1 (MCP-1) (BD Bioscience Pharmingen, San Jose, CA, USA) or CD68 (DakoCytomation, Glostrup, Denmark), a marker of monocytes and macrophages. The number of CD68-positive cells was counted with a light microscope (×200, 10 fields per rat, n = 6 in each group). To detect capillary endothelial cells, immunohistochemical staining of the sections was performed with a rabbit polyclonal antibody raised against von Willebrand factor (vWF, DakoCytomation). The number of capillary vessels was counted using a light microscope (×200, 10 fields per rat, n = 6 in each group).

2.5. Western blot analysis

Western blot was performed as previously described [18]. Briefly, LV tissues were homogenized in 0.1% Tween-20 with a protease inhibitor, loaded (40 μg) on a 7.5% sodium dodecyl sulfate-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking for 2 h, membranes were incubated with MMP-2 (Laboratory Vision, Fremont, CA, USA) or MMP-9.
Chemicon, Temecula, CA, USA) rabbit polyclonal antibodies (1:200), then incubated with peroxidase labeled with secondary antibody (1:1000). Positive protein bands were visualized with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and measured by densitometry. A mouse polyclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control (n=5 in each group).

2.6. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Heart tissues (n=5 in each group) were homogenized with TissueLyser (Qiagen, Hilden, Germany). Total RNA was extracted using RNeasy Mini Kit (Qiagen), followed by reverse transcription into cDNA using the avian myeloblastosis virus transcriptase (Ambion, Austin, TX, USA), according to the manufacturers’ protocol. PCR amplification was performed in 50 μl containing 1 μl of cDNA and 25 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The following sequence-specific primers were used for TGF-β, as described previously[19]: forward, 5′-GTTCTTCAATACGTCAGACATTCG-3′; reverse, 5′-CATTATCTTTGCTGTCACAAGAGC-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA amplified from the same samples was served as an internal control: forward, 5′-GAACATCATCCCTGCATCCA-3′; reverse, 5′-CCAGTGAGCTTCCCGTTCA-3′. After an initial denaturation at 95 °C for 10 min, a 2-step cycle procedure was used (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). The data were analyzed with Sequence Detection Systems software.

Fig. 1. Pathological findings in acute myocarditis after AM infusion. A: Representative H&E staining of myocardial sections showed markedly decreased inflammation and tissue necrosis in AM-treated hearts as compared to control hearts. Insets are transverse sections of myocardial freewall. B: Semi-quantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were significantly lower in AM-treated hearts as compared to control hearts (n=8 in each group). Sham tissues exhibited no measurable pathological changes. Data are mean±S.E. *, P<0.05 vs. control. C: Representative picrosirius staining showed decreased collagen deposition in AM-treated hearts as compared to control hearts. D: Collagen volume fraction in 10 random representative fields (×200) confirmed a significant decrease in AM-treated hearts vs. control hearts (n=5 in each group). Data are mean±S.E. *, P<0.05 vs. sham; †, P<0.05 vs. control.

Fig. 2. Infiltration of inflammatory cells in myocardium. A: Immunohistochemical analysis of CD68-positive cell infiltration in myocardium. AM infusion markedly attenuated the increase in CD68-positive cells in myocarditic hearts. Scale bars: 50 μm. B: Semi-quantitative analysis of CD68-positive cell infiltration. CD68-positive cells in 10 random representative high-power fields (×200) confirmed a significant decrease in AM-treated hearts vs. control hearts (n=6 in each group). Data are mean±S.E. *, P<0.05 vs. control.
2.7. Enzyme-linked immunosorbent assay (ELISA)

To investigate the effect of AM infusion on serum MCP-1 level, blood was drawn from the heart before excision \((n=6\) in each group). Blood was centrifuged and serum samples were frozen and stored at \(-80 \, ^\circ\text{C}\). Serum MCP-1 level was measured by ELISA according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

Fig. 3. Effects of AM infusion on MCP-1 expression. A: Representative myocardial sections immunohistochemically stained with anti-MCP-1 antibody showed increased vascular endothelial and myocyte staining of MCP-1 (arrows) and the presence of giant cells (arrowheads) in control hearts as compared to AM-treated hearts. Sham hearts showed subtle endothelial staining. Scale bars: 20 \(\mu\)m. B: Serum MCP-1 level was greatly increased in myocarditic rats. However, the increase in serum MCP-1 was significantly attenuated by AM infusion \((n=6\) in each group). Data are mean±S.E. *, \(P<0.05\) vs. sham; †, \(P<0.05\) vs. control.

Fig. 4. Effects of AM infusion on MMP and TGF-\(\beta\) expression. A and B: Western blot analysis for MMP-2 (A) and -9 (B) expression. Levels of MMP-2 and -9 were significantly increased in control hearts. MMP-2 expression was markedly decreased by AM infusion, and MMP-9 expression tended to be decreased after AM infusion \((n=5\) in each group). C: Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) for TGF-\(\beta\) expression. Expression of TGF-\(\beta\) was increased in myocarditis and significantly decreased by AM treatment \((n=5\) in each group). Data are mean±S.E. *, \(P<0.05\) vs. sham; †, \(P<0.05\) vs. control.
2.8. Hemodynamic study

Hemodynamic measurements were taken on day 21 post-myosin injection \((n=7\) in each group). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium \((30 \text{ mg/kg})\) as a supplement to maintain mild anesthesia. A 1.5 Fr micromanometer-tipped catheter \((\text{Millar Instruments, Houston, TX, USA})\) was advanced into the left ventricle through the right carotid artery, and a polyethylene catheter \((\text{PE-50})\) was advanced into the right ventricle through the right jugular vein to measure right ventricular pressure. Heart rate was also monitored by electrocardiography. As hemodynamic indices, heart rate, mean arterial pressure, LV end-diastolic pressure, maximum \(dP/dt\), and minimum \(dP/dt\) were used.

2.9. Echocardiography

Echocardiography was performed on day 21 post-myosin injection \((n=7\) in each group). A 12-MHz probe was placed in the left 4th intercostal space for M-mode imaging using 2D echocardiography \((\text{Sonos 5500, Philips, Bothell, WA, USA})\). M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular \((\text{LV})\) end-diastolic and end-systolic dimension, LV fractional shortening \((\text{FS})\), and LV ejection fraction \((\text{EF})\) were measured in three consecutive cardiac cycles by the American Society for Echocardiology leading-edge method \((n=10\) in each group). EF and FS were calculated from the following formula, respectively:

\[
\text{EF} = \frac{\text{end-diastolic volume} - \text{end-systolic volume}}{\text{end-diastolic volume}}
\]

\[
\text{FS} = \frac{\text{end-diastolic diameter} - \text{end-systolic diameter}}{\text{end-diastolic diameter}}
\]

2.10. Statistical analysis

All data were expressed as mean±S.E. Comparisons of parameters among the groups were made by one-way ANOVA. Table 1

<table>
<thead>
<tr>
<th>Physiological profiles of three experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td><strong>Body weight, g</strong></td>
</tr>
<tr>
<td><strong>Ventricular weight, g</strong></td>
</tr>
<tr>
<td><strong>Lung/body weight</strong></td>
</tr>
<tr>
<td><strong>Heart rate, bpm</strong></td>
</tr>
<tr>
<td><strong>MAP, mm Hg</strong></td>
</tr>
<tr>
<td><strong>LVSP, mm Hg</strong></td>
</tr>
<tr>
<td><strong>LVEDP, mm Hg</strong></td>
</tr>
</tbody>
</table>

Sham, sham rats given vehicle; Control, myosin-treated rats given vehicle; AM, myosin-treated rats given AM; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. Data are mean±S.E. *\(P<0.05\) vs. sham; †\(P<0.05\) vs. control. \(n=7\) in each group.
ANOVA, followed by Newman–Keuls’ test. Comparisons of parameters between two groups were made by Student’s t-test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Histopathological improvement after AM infusion

Sections of left ventricular tissue demonstrated substantial myocardial necrosis, infiltration of inflammatory cells and edema in the control group, which was significantly limited primarily to areas directly adjacent to arterial vessels with AM treatment (Fig. 1, panel A). Blinded histological grading confirmed decreased myocyte necrosis, granulation, inflammation and tissue edema in the AM group as compared in the control group (Fig. 1, panel B). Picrosirius red staining revealed increased collagen deposition in the control group (Fig. 1, panel C). However, AM infusion attenuated collagen deposition in the myocardium (Fig. 1, panel D).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Echocardiographic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>4.9±0.1</td>
</tr>
<tr>
<td>LVDs, mm</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>EF, %</td>
<td>75±1</td>
</tr>
</tbody>
</table>

Sham, sham rats given vehicle; Control, myosin-treated rats given vehicle; AM, myosin-treated rats given AM; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; EF, ejection fraction. Data are mean±S.E. *$P<0.05$ vs. sham; †$P<0.05$ vs. control. $n=10$ in each group.
3.2. Infiltration of CD68-positive cells in myocardium

A significant decrease in infiltration of CD68-positive inflammatory cells was observed in the AM group as compared to the control group (790 ± 80 vs. 1468 ± 109 cells/mm²; Fig. 2, panel A and B). Sham tissues showed little or no myocardial CD68 positivity (data not shown).

3.3. Expression of MCP-1 after AM infusion

The expression of MCP-1 was increased in myocarditis; it was localized to the vascular endothelium and also in myocytes surrounding and adjacent to the areas of inflammation (Fig. 3, panel A). Heart sections in the AM group showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in the control group, whereas a significant decrease was observed in the AM group (Fig. 3, panel B).

3.4. Effects of AM infusion on MMPs and TGF-β expression

Western blotting analysis revealed that myocardial levels of MMP-2 and -9 were significantly increased in the control group. MMP-2 expression was markedly decreased by AM infusion, and MMP-9 expression tended to be decreased after AM infusion (Fig. 4, panel A). Quantitative real-time RT-PCR analysis demonstrated increased expression of TGF-β in the heart of the control group which was significantly attenuated by AM treatment (Fig. 4, panel B). AM infusion did not significantly influence cardiac expression of IL-1β and TNF-α (data not shown).

3.5. Angiogenesis induced by AM infusion

To determine the effect of AM treatment on angiogenesis, vWF-stained heart sections were subjected to capillary density counting. Capillary density was increased in the control group, particularly in areas directly adjacent to tissue necrosis (1146 ± 57 vs. 782 ± 21 cells/mm², Fig. 5, panel A and B). However, in AM-treated tissues, capillary density was further significantly increased not only in the peri-necrotic areas but also in apparently healthy myocardium (1347 ± 82 vs. 1146 ± 57 cells/mm²), suggesting that stimulation of angiogenesis was further augmented by AM treatment.

3.6. Heart weight and hemodynamics after AM infusion

The physiological and catheter-derived functional properties on day 21 post-myosin injection are summarized in Table 1 and Fig. 6, panel A. Myocarditic hearts showed significantly increased heart weight to body weight ratio, which was decreased by AM treatment. AM treatment also significantly improved maximum dP/dt. For both minimum dP/dt and LVEDP, we did not find significant differences. On echocardiography, AM administration significantly attenuated increased wall thickness after acute myocarditis. AM significantly improved LV fractional shortening and ejection fraction, although LVDd did not significantly differ between control and AM groups (Table 2 and Fig. 6, panel B and C).

4. Discussion

In the present study, AM treatment showed the following effects in acute myocarditis: 1) reduced necrosis, inflammation and edema in the myocardium; 2) attenuated expression of MCP-1, MMP-2 and TGF-β; 3) increased capillary density suggestive of angiogenesis; and 4) improved cardiac function.

This experimental autoimmune myocarditis model is triphasic, consisting of an antigen priming phase from days 0 to 14, an autoimmune response phase from days 14 to 21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [20]. MCP-1 expression is increased in the heart from days 15 to 27 post-myosin injection, and serum MCP-1 level is elevated from days 15 to 24 [21]. We treated rats with AM at 1 week after myosin injection, corresponding to an early time point in the disease process. Pathological examination demonstrated that infusion of AM attenuated myocyte necrosis and inflammation in acute myocarditis. This observation was supported by a decrease in infiltration of CD68-positive inflammatory cells in the myocardium. Interestingly, both MCP-1 expressions in the myocardium and serum MCP-1 level were decreased after AM infusion. MCP-1 is a member of the C–C subfamily of chemokines with chemotactic activity for major inflammatory cells such as monocytes and T lymphocytes [22], and this model of acute myocarditis has previously been shown to be associated with MCP-1 [21]. Thus, the decrease in CD68-positive cell infiltration in the myocardium following this treatment may be attributable to inhibition of MCP-1 production by AM. The inhibitory effect of AM on MCP-1 expression is consistent with a previous in vitro study showing that AM inhibited pressure-induced MCP-1 expression in mesangial cells [23]. Recently, it has been demonstrated that AM has anti-inflammatory effects through modulation of macrophage migration inhibitory factor secretion [24]. Importantly, overexpression of MCP-1 induces myocarditis and subsequent development of heart failure [25]. These findings suggest that the inhibitory effect on MCP-1 expression and subsequent anti-inflammatory effect of AM are possible mechanisms of the improvement in acute myocarditis.

We found a significant increase in heart weight to body weight ratio and wall thickness 3 weeks after myosin injection. These results indicate exaggerated edematous changes in myocarditic hearts. Infusion of AM reduced overall heart weight to body weight ratio and wall thickness in myocarditic hearts and attenuated histological edematous changes. Earlier studies have demonstrated that AM decreases vascular congestion and endothelial hyperpermeability in the heart [11], reduces hyperpermeability of cultured endothelial cells and inhibits pulmonary edema [26]. Thus, it is interesting to speculate that the attenuation of edematous changes in the
Adrenomedullin (AM) may be attributable to reduction of endothelial hyperpermeability by AM.

In the present study, AM infusion significantly increased the capillary density in myocarditic hearts. In fact, earlier studies have demonstrated angiogenic properties of AM in vitro and in vivo [27–29]. Importantly, improvement in myocardial vascular supply has been shown to decrease necrosis and inflammation in viral myocarditis [30,31]. These results suggest that AM-induced angiogenesis in the myocardium may be responsible for the improvement in acute myocarditis, which was indicated by reduced necrosis and inflammation in myocarditic hearts.

As previously mentioned, experimental autoimmune myocarditis chronically develops into a dilated cardiomyopathy phenotype [20]. MMPs have been associated with left ventricular remodeling [32] and here we showed increased expression of MMP-2 and -9 as well as increased collagen deposition in myocarditic hearts. In the present study, AM treatment significantly reduced both MMP-2 expression and collagen deposition. In addition, our observation demonstrated that the expression of TGF-β, a profibrogenic factor, was also attenuated by AM treatment. It has been demonstrated that AM decreases the expression of TGF-β in experimental mesangioproliferative glomerulonephritis [33]. These results suggest that AM may have beneficial effects on myocardium, possibly through regulation of factors involved in LV remodeling. In the present study, LVDd did not significantly differ between the control and AM groups. However, it should be noted that AM significantly reduced wall thickness possibly due to reduction of myocardial edema, leading to a slight increase in the inner diameter of the LV and a significant increase in ejection fraction. The major effect of AM was to reduce myocardial edema but not remodeling, despite reducing biochemical markers of remodeling.

Earlier studies have shown that short-term infusion of AM decreases arterial pressure and increases cardiac output in patients with acute heart failure [5]. These findings suggest that the improvement in cardiac function after acute myocarditis may be mediated partly by the hemodynamic effects of AM. However, despite the well-characterized vasorelaxant properties of AM [4], there was a significant increase in mean arterial pressure after AM treatment in our model. These findings suggest that AM induced limited direct hemodynamic action. Taking these findings together, the improvement of cardiac function after AM treatment may have been mediated by the improvement of pathological findings including necrosis, inflammation and edema in the myocardium rather than by AM-induced hemodynamic effects.

In conclusion, infusion of AM improved cardiac function and pathological findings including inflammatory infiltration and edema in a rat model of acute myocarditis. The beneficial effects of AM may occur at least in part by inhibitory effects on MCP-1, MMP-2 and TGF-β, and by enhancement of angiogenesis after acute myocarditis. Thus, infusion of AM may be a potent therapeutic strategy for acute myocarditis.

Acknowledgements

This work was supported by research grants for Cardiovascular Disease (16C-6 and 17A-1) and Comprehensive Research on Aging and Health from the Ministry of Health, Labour and Welfare, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); and Health and Labor Sciences Research Grants (Human Genome Tissue Engineering 009).

References


