Expression of Coxsackievirus and Adenovirus Receptor in Hearts of Rats With Experimental Autoimmune Myocarditis

Masahiro Ito, Makoto Kodama, Masayoshi Masuko, Masayuki Yamaura, Koichi Fuse, Yumiko Uesugi, Satoru Hirono, Yuji Okura, Kiminori Kato, Yuko Hotta, Takao Honda, Ryozo Kuwano, Yoshifusa Aizawa

Abstract—The expression of coxsackievirus and adenovirus receptor (CAR) was dominant in the brains and hearts of mice until the newborn phase. There is no detailed information concerning the relation between the expression of CAR and development of hearts. It is also uncertain whether CAR is able to be induced in adult hearts after cardiac injury. We demonstrated that CAR was abundant in the hearts of newborn rats but was barely detectable in the hearts of adult rats. The expression of CAR in rat hearts with experimental autoimmune myocarditis, which was induced by immunization of purified cardiac myosin, was serially investigated. Active myocarditis was observed from day 15 after immunization. By immunohistochemistry, cardiomyocytes were strongly stained for CAR antibody from days 24 to 42. CAR mRNA was also detected from days 18 to 30 by using reverse transcription–polymerase chain reaction. In the next experiment, the induction of CAR on isolated cardiomyocytes was investigated. CAR was barely detectable in cultured cardiomyocytes by Western blot analysis after isolation. This molecule gradually appeared along with the creation of clusters and beating of cardiomyocytes. Furthermore, the induction of CAR in cultured cardiomyocytes increased after supplement with conditioned medium of rat splenocytes activated by concanavalin A. In conclusion, rat CAR is expressed strongly in the hearts of newborn rats and is suppressed in those of adult rats. The expression of CAR is enhanced during the active phase of experimental autoimmune myocarditis and is induced by inflammatory mediators. CAR may play a role in cell-to-cell contact and adhesion of cardiomyocytes. (Circ Res. 2000;86:275-280.)

Key Words: coxsackievirus and adenovirus receptors ■ myocarditis ■ cardiomyocytes

Coxsackievirus and adenovirus receptor (CAR) was recently identified, and cDNA of this molecule was isolated in humans and mice.1–3 CAR exhibits both tissue- and species-specific expression. In mice, CAR was present in the brain, liver, heart, lungs, and kidneys.1,2 In our previous study, CAR was strongly expressed in the brains and hearts of mice until the newborn phase; its expression then decreased and subsequently became undetectable in adult mice.4 These observations imply that the expression of CAR exhibits developmental changes and that this molecule may play roles in differentiation and development of the brain and heart.

CAR structurally belongs to the immunoglobulin superfamily, similar to intercellular adhesion molecule-1 (ICAM-1) and neural cell adhesion molecule (N-CAM). Both ICAM-1 and N-CAM have been demonstrated to be related to either the normal development of the heart or the pathogenesis of cardiovascular diseases.5–7 The physiological functions of CAR have not yet been elucidated.3 Whether CAR plays a role in left ventricular remodeling after myocardial injury has not yet been determined. In the present study, we demonstrated that CAR was reexpressed in the hearts of adult rats with rat experimental autoimmune myocarditis, which is an animal model of human giant cell myocarditis leading to dilated cardiomyopathy. Furthermore, we examined the expression of CAR in cultured rat cardiomyocytes supplemented with conditioned medium of rat splenocytes activated by concanavalin A (Con A) to elucidate the mechanisms of the induction of CAR, accompanied by inflammation.

Materials and Methods

Immunization

Experimental autoimmune myocarditis was elicited in Lewis rats by immunization of purified pig cardiac myosin as previously described.8 On day 0, 15 rats were immunized with antigen-adjuvant emulsion. Another group of 15 rats was immunized with saline as the control.

Sampling

Newborn rats were killed on day 1 after birth, and their hearts were removed. Myosin-immunized rats were killed on days 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 49, and 56 after immunization. Macroscopic findings were scored in 3 grades as previously described.8 Hearts were then removed and weighed to calculate the...
heart weight/body weight ratio. The ventricular muscle was used for the following analysis.

Preparation of Antibody
Seventeen amino acid residues (KTQYNQVPSEDPERAPQ) in an intracellular domain of CAR were selected. Antibodies against CAR were prepared as previously described.9

Histopathology and Immunohistochemistry
CAR was immunohistochemically detected by use of purified rabbit anti-murine CAR antibodies. Positive findings for CAR produced a brown color. The severity of inflammation was graded from 0 (no inflammation) to 3 (severe inflammation) as previously described.10 The expression of CAR was graded as follows: −, negative; ±, weakly positive; +, moderately positive; and ++, strongly positive.

RT-PCR Analysis
Total RNA was extracted from rat hearts, and reverse transcription (RT)-polymerase chain reaction (PCR) was performed with primers (ATGGATCCTACACCCGAACAGAGGATCG [sense] and GC-GAATTCCGTCGCGACTTGACAT [antisense]). β-Actin cDNA was amplified as the control. Thirty-three cycles of amplification reactions (94°C denaturation, 53°C annealing, and 72°C extension) were performed.

Preparation of Conditioned Medium of Rat Splenocytes Activated by Con A
Single-splenocyte suspensions of adult rat11 were cultured for 2 days in the presence of 10 μg/mL of Con A, and the supernatant was obtained (Con A medium).

Preparation of Cultured Cardiomyocytes
Cardiomyocytes were prepared from neonatal rat ventricles by the modified method of Libby.12 The cardiomyocytes were divided into 4 groups. Group 1 was not cultured. Group 2 was cultured for 24 hours. Groups 3 and 4 were cultured for 96 hours. Group 4 was cultured in standard medium for an initial 24 hours and then cultured in Con A medium (10% of culture medium) for 72 hours.

Western Blot Analysis
Total protein was prepared from the left ventricle of 1-day-old and adult rats and from cultured cardiomyocytes with cell lysis buffer. Protein (15 μg) from total cell lysates13 was separated by SDS-PAGE and analyzed by immunoblotting with the polyclonal anti-CAR antibodies. The bands of CAR were analyzed with NIH Image and expressed in relation to the control values.

Statistical Analysis
Data are presented as mean±SD. Statistical assessment of the significance among groups was made by 1-way ANOVA followed by the Bonferroni-Dunn method. A value of P<0.05 was considered significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
Expression of CAR in Hearts of Newborn and Adult Rats
To confirm that the PCR product was an amplified fragment of rat CAR cDNA, the PCR product on day 21 was directly sequenced. Because the sequence of the PCR product had a homology of 93% with murine CAR, we interpreted this product as rat CAR (sequence not shown). RT-PCR revealed that CAR mRNA was expressed abundantly in the hearts of newborn rats, whereas only a slight expression of CAR was detected in the hearts of normal adult rats (Figure 1).

Immunohistochemical staining showed that the expression of CAR was strong in the hearts of newborn rats but was undetectable in those of adult rats (Figure 2).

Expression of CAR in Rat Hearts With Myocarditis
Clinical Course of Myosin-Immunized Rats
One of the myosin-immunized rats died on day 18, during the active phase of myocarditis. This rat showed macroscopic evidence of severe myocarditis and was thought to have died of heart failure. No control rats died spontaneously throughout the period of this experiment (Table).

Macroscopic Findings
The extent of discolored areas of the hearts and the appearance of pericardial effusion are summarized in the Table.

Figure 1. CAR mRNA in hearts of newborn and adult rats detected by RT-PCR. Top bands represent the expression of CAR in the myocardium. Lower bands represent β-actin mRNA.

Figure 2. Immunohistochemical staining for CAR. A, Newborn rat. The expression of CAR was strong. Vascular wall was not stained for CAR. B, Adult rat. Cardiomyocytes were not stained for CAR. Magnification ×200.
From the 15th day after immunization, discolored areas were observed on the cardiac surface of the myosin-immunized rats. Hearts of the myosin-immunized rats were markedly enlarged, as shown by heart weight/body weight ratios (Table). Pericardial effusion was observed in myosin-immunized rats on days 18, 21, and 24. Histological Study

Microscopic findings are summarized in the Table. There were no microscopic abnormalities in the hearts of either the control rats or the myosin-immunized rats on day 9. Small lesions of mononuclear cell infiltration were observed in the hearts of the myosin-immunized rats on day 12. Thereafter, inflammatory lesions spread extensively, and interstitial edema became evident. From day 21, in addition to mononuclear cell infiltration, myocardial necrosis was observed. Fibrosis became prominent on day 36. Most of the infiltrated cells disappeared on day 49, and the inflammatory lesions were replaced by fibrosis.

RT-PCR Analysis

CAR mRNA was identified in the hearts of the myosin-immunized rats on days 18 to 30 and peaked on days 21 and 24. CAR mRNA was not detectable after day 33 by RT-PCR (Figure 3). CAR mRNA was not detected in the control group throughout the experiment by our RT-PCR (data are not shown).

Immunohistochemical Analysis

Immunohistochemical staining for CAR was negative in the hearts of control rats. Hearts of the rats with experimental autoimmune myocarditis were stained with anti-CAR antibody from day 24. Immunoreactivity of CAR was found in the cardiomyocytes of rats with experimental autoimmune myocarditis, not only in the inflammatory lesions but also in the intact area, where infiltration cells and myocardial degeneration were not found. Scar tissue and the vascular wall were not stained. The staining was most intense in the hearts obtained on day 33, after which immunoreaction for CAR gradually decreased (Figure 4).

Induction of CAR in Cultured Cardiomyocytes

Although CAR was expressed abundantly in the hearts of the newborn rats, only a slight expression of CAR was detected in cultured rat cardiomyocytes after isolation from the newborn rats by Western blot analysis (Figure 5A). The expression of CAR was suppressed after isolation, but then it gradually increased according to the creation of clusters and beating of cardiomyocytes. Furthermore, the induction of CAR was more significantly enhanced in cultured cardiomyocytes treated with Con A medium than those treated with culture medium (Figure 5B).

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**Clinical Course of Control and EAM in Individual Rats**

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<th>9</th>
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<th>15</th>
<th>18</th>
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EAM indicates experimental autoimmune myocarditis; BW, body weight.

*0 indicates normal; 1, a focal discolored area present; and 2, multiple or diffuse discolored areas present on the cardiac surface.

†0 indicates no inflammation; 1, presence of a few small lesions, not exceeding 0.25 mm² in size; 2, presence of multiple small lesions or a few moderately sized lesions, not exceeding 6.25 mm²; and 3, the presence of multiple moderately sized lesions or larger lesions.
CAR structurally belongs to the immunoglobulin superfamily, and its physiological function has not yet been clarified. The immunoglobulin superfamily includes many cell-surface proteins that mediate cell-to-cell recognition or antigen recognition in the immune system. N-CAM, which also belongs to the immunoglobulin superfamily, was expressed prominently in the embryonic phase, and its expression was suppressed in the hearts of adult rats.\textsuperscript{14} N-CAM immunoreactivity was reexpressed in transplanted extrinsically denervated human hearts and also in the hypertrophic myocardium of rats.\textsuperscript{15,16} From these observations, N-CAM was considered to play roles in both development of the heart and innervation to the heart. The expression of CAR was high in the

**Discussion**

CAR structurally belongs to the immunoglobulin superfamily, and its physiological function has not yet been clarified. The immunoglobulin superfamily includes many cell-surface proteins that mediate cell-to-cell recognition or antigen recognition in the immune system. N-CAM, which also belongs to the immunoglobulin superfamily, was expressed prominently in the embryonic phase, and its expression was suppressed in the hearts of adult rats.\textsuperscript{14} N-CAM immunoreactivity was reexpressed in transplanted extrinsically denervated human hearts and also in the hypertrophic myocardium of rats.\textsuperscript{15,16} From these observations, N-CAM was considered to play roles in both development of the heart and innervation to the heart. The expression of CAR was high in the

**Figure 4.** Immunohistochemical studies of CAR in myocardium of rat hearts with experimental autoimmune myocarditis. A, Day 12. A large number of mononuclear cells were evident in the myocardium. Cardiomyocytes were generally intact. Cardiomyocytes were not stained with anti-CAR antibody. B, Day 24. Inflammatory cell infiltration and fragments of degenerated myocardial fibers were observed. Minimal immunoreactivity was observed in cardiomyocytes. C, Day 33. Extensive infiltration of inflammatory cells and necrosis of cardiomyocytes were observed. Cardiomyocytes were strongly stained, but vascular wall and inflammatory cells were not stained with anti-CAR antibody. D, Day 36. Fibrosis was strongly detected. Myocytes were still stained, whereas scar tissues were not stained with anti-CAR antibody. E, Day 49. Fibrosis was prominent in this period. Immunoreactivity for CAR decreased. Scar tissues were not stained. Magnification $\times$200.
cardiomyocytes after isolation from these hearts. CAR gradually appeared again as cardiomyocytes began to make clusters and start a beat. On the basis of these results, we suggest that CAR may play roles in cell-to-cell contact and to the adhesion of cardiomyocytes and act on myocardial regeneration.

Rat experimental autoimmune myocarditis is inducible by purified cardiac myosin and is an animal model of human giant cell myocarditis, which leads to dilated cardiomyopathy. T cells play an important role in inducing myocarditis in this model. We examined the expression of CAR in experimental autoimmune myocarditis of rats to clarify whether CAR was reenhanced in diseased hearts. In the course of experimental autoimmune myocarditis, the expression of CAR in the hearts was low or undetectable before the onset of the disease, and then its expression became apparent during the active phase of myocarditis. Next, the expression of CAR decreased in the chronic phase. CAR, which belongs to the immunoglobulin superfamily, was also induced in myocardial cells of mice with acute viral myocarditis. Its expression coincided with cell infiltration. CAR was demonstrated to be associated with cell-mediated myocardial injuries by activated lymphocytes. On the other hand, the expression of rat CAR was preceded by several days of massive cell infiltration. Accordingly, the expression of CAR in myocardial cells might not be related to myocardial injuries in acute myocarditis. CAR may be associated with the healing phase of myocarditis or regeneration of damaged myocardium.

The mechanism responsible for the induction of CAR had not been elucidated. In the present study, CAR was induced when myocytes were treated with conditioned medium of rat splenocytes activated by Con A. This result implied that the expression of CAR was induced by inflammatory mediators. We previously revealed the time course of the expression of various cytokine mRNA in experimental autoimmune myocarditis. mRNA of proinflammatory cytokines, such as interferon-γ, tumor necrosis factor-α, and interleukin-1β, and inducible nitric oxide synthase were expressed in the myocardium of experimental autoimmune myocarditis from the beginning of the acute inflammatory phase. CAR mRNA was reexpressed after the expression of these cytokines. The present study suggests that inflammatory mediators play a role in the induction of CAR in experimental autoimmune myocarditis. Further study is necessary to identify the specific mediators inducing CAR.

In conclusion, the expression of CAR was enhanced in experimental autoimmune myocarditis. Our results suggest that CAR is induced by inflammatory mediators. This molecule may contribute to cell-to-cell contact and to the adhesion of cardiomyocytes and act on myocardial regeneration.

References


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Materials and Methods

Experimental Animals

Female Lewis rats (8 weeks old) and pregnant Lewis rats were purchased from Charles River Japan Inc. (Atsugi, Japan) and were maintained in our animal facilities.

Immunization

Cardiac myosin was purified from the ventricular muscle of pig hearts according to the procedure previously described, and used for creation of rats with autoimmune myocarditis as an antigen. The antigen was dissolved at a concentration of 20 mg/mL in PBS containing 0.3 mol/L KCl and mixed with an equal volume of Freund's complete adjuvant (FCA) containing 10 mg/mL of Mycobacterium tuberculosis (Difco Laboratories). On Day 0, fifteen rats were immunized with 0.2 mL of antigen-adjuvant emulsion. Fifteen rats were immunized with 0.1 mL of saline mixed with an equal volume of FCA and used as the control.

Sampling

Newborn rats were sacrificed under ether anesthesia on Day 1 after birth and their hearts were removed. Myosin-immunized rats were sacrificed under ether anesthesia on Days 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 49 and 56 after immunization. At thoracotomy, macroscopic findings were scored according to the following grades: 0, normal; 1, a focal discolored area present; and 2, multiple or diffuse discolored areas present on the cardiac
surface. Hearts were then removed and immediately weighed to calculate the heart weight-to-body weight ratio. Then, a part of the ventricular muscle was frozen for analysis of mRNA. The remainder was fixed in Bovin's liquid (picric acid: formaldehyde: acetic acid =15: 5: 1).

Preparation of Antibody

Because CAR belongs to the single-pass transmembrane protein, 17 amino acid residues in an intracellular domain were selected for preparation of antibody. A peptide (KTQYNQVPSEDFERAPQ) was synthesized with an additional cysteine at the C-terminus, conjugated to keyhole limpet hemocyanin (Calbiochem - Novabiochem), and was used to immunize a white rabbit. The resulting antiserum was purified by an immunoabsorbent affinity chromatography on a CNBr-activated Sepharose 4B (Pharmacia) column coupled with the peptide.9

Histopathology and Immunohistochemistry

The removed hearts were fixed in Bovin's liquid. Fixation lasted about 24 hours at room temperature, then the fixed samples were embedded in paraffin, and sliced at several levels to gain transverse sections for immunohistochemical studies. Sliced sections (5 μm) of the hearts were washed in PBS, and were incubated with 10% normal goat serum (IBL) for 30 minutes at room temperature. The tissue sections were then incubated in 5% goat serum containing 1% of purified rabbit anti-murine CAR antibodies for 1 hour, followed by incubation with biotinylated goat anti-rabbit IgG (IBL)
diluted to 1:100 for 1 hour. The samples were subsequently incubated in horseradish peroxidase conjugated streptavidin (IBL) diluted to 1:80 for 1 hour. The reaction products were visualized using 0.5% diaminobenzidine and H$_2$O$_2$. The sections were counterstained with hematoxylin. Positive immunohistochemical staining for CAR produced a brown color. The severity of inflammation was graded as follows: 0, no inflammation; 1, presence of a few small lesions, not exceeding 0.25 mm$^2$ in size; 2, presence of multiple small lesions or a few moderately sized lesions, not exceeding 6.25 mm$^2$; and 3, the presence of multiple moderately sized lesions or larger lesions.$^{10}$ The expression of CAR was graded as follows: -, negative; $\pm$, weakly positive; $+$, moderately positive; and $++$, strongly positive.

**RT-PCR analysis**

Total RNA was extracted from rat hearts using acid guanidium thiocyanate-phenol-chloroform. cDNA was synthesized from 5 $\mu$g of total RNA with random primers and Avian Myeloblastosis Virus reverse transcriptase (Takara Shuzo) in a final volume of 20 $\mu$l. Primers of the rat CAR spanned nucleotides 160 to 360 of the murine cDNA because the rat CAR sequence was not available. Two primers, ATGGATCCTACACCCGAACAGAGGATCG (sense) and GCGAATTCGCCTGACGACCTGACAT (antisense), were chosen. The primers, ATCATGTGGAGACCATCTTCA (sense) and CATCTCTTGCTGAAGTCCA (antisense), were chosen for amplification of
β-actin cDNA as the control. Amplification reactions were carried out with 1 μg of each primer and 1.5 U of recombinant Taq DNA polymerase (Takara Shuzo). The samples were placed in a thermocycler (Takara PCR thermal cycler MP) using 94 °C denaturation, 53 °C annealing, and 72 °C extension temperatures for one cycle. Thirty-three cycles were performed, and amplified products were separated on agarose gels and stained with ethidium bromide.

**Preparation of conditioned medium of rat splenocytes activated by Con A (Con A-medium)**

Spleens were removed from adult rats, and single-cell suspension was prepared as we previously described. Spleen cells were suspended at a density of 5×10^6 cells/mL in RPMI-1640 and cultured for 2 days in the presence of 10 μg/ml of concanavalin A (Con A) (Sigma Chemical, St. Louis). The cells were removed by centrifugation and the supernatant was obtained (Con A-medium).

**Preparation of cultured cardiomyocytes**

Cardiomyocytes were prepared from neonatal rat ventricles by a modified Libby's method. Hearts were removed from 1-day-old newborn rats, minced in calcium-free PBS, and digested in PBS contained trypsin (Difco Laboratories, 0.3mg/ml) and collagenase (Type II, Sigma Chemical, 0.3mg/ml). The expression of CAR was examined in cardiomyocytes isolated from hearts of the newborn rats. The isolated cardiomyocytes were dispersed into
plastic dishes for 1 hour to permit attachment and selective removal of fibroblasts, and transferred to tissue culture dishes (Becton Dickinson). The cardiomyocytes were divided into four groups designated as 1, 2, 3, and 4. The cardiomyocytes of Group 1 were collected for Western blot analysis. The cardiomyocytes of Groups 2, 3, and 4 were cultured with 5% fetal bovine serum, Ham's medium F12 (ICN Biomedicals Inc, Ohio), Hanks' balanced salt solution (Sigma Chemical CO, St. Louis), and a penicillin-streptomycin mixture for 24 hours at 37 °C in a humidified 5 % CO₂/95 % air incubator. The cardiomyocytes of Group 2 were collected for experiment. Groups 3 and 4 were replaced with fresh culture media after 24 hours, and were proceeded to culture in the absence (Group 3) or presence (Group 4) of Con-A medium (10% of culture medium). The cardiomyocytes of these 2 groups were collected for Western blot analysis after 72 hours.

**Western Blot Analysis**

Total protein was prepared from the left ventricle of 1-day-old and adult rats, and from cultured cardiomyocytes with cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA and 1 mM sodium orthovanadate) containing 10 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/mL leupeptin. Cell lysates (15 µg) were solubilized in Laemmli sample buffer, then boiled for 3 minutes at 100 °C. The protein from total cell lysates was separated by SDS-polyacrylamide gel electrophoresis (7.5% gel) and analyzed by
immunoblotting. The blots were electrophoretically transferred to polyvinilidene difluoride (PVDF; Immobilon-P; Millipore, Bedford, MA) in blocking buffer (TBS; 20 mM Tris, 137 mM NaCl, pH 7.5). After extensive washing, these preparations were incubated with the polyclonal anti-CAR antibodies (1:600 dilution) in the blocking buffer at 4 °C. The transferred polyvinilidene difluorides were then further washed three times with TBS-T (20 mM Tris, 137 mM NaCl, 0.05% Triton-X 100, pH 7.5). The binding of antibodies was detected using horseradish-paraoxidase-conjugated sheep anti-mouse IgG (Amersham, Little Chalfont, UK) and visualized with ECL chemiluminescence reaction reagents and ECL-hyperfilm (Amersham, Littel Chalfont, UK). The bands of CAR were analyzed with a NIH Image Version 1.62 and expressed in relation to the control values.

Statistical Analysis

Data are presented as mean ± SD. Statistical assessment of the significance among groups was made by one-way ANOVA followed by the Bonferroni-Dunn method. A value of P<0.05 was considered significant.