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Original article

The coxsackie–adenovirus receptor induces an inflammatory cardiomyopathy independent of viral infection

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ABSTRACT

The coxsackie–adenovirus receptor (CAR) is a viral receptor for Group B coxsackieviruses (CVBs) and adenoviruses. CAR has been linked with the innate immune response to CVB myocarditis, and with activation of inflammatory cells *in vitro*. We hypothesized that CAR activates signals that promote inflammation in the myocardium independent of viral infection. To test this we conditionally overexpressed murine CAR in cardiomyocytes of adult binary transgenic mice under the control of a tetracycline-responsive (tet-off) α-myosin heavy chain (αMtTA) promoter (mCAR$^+$/αMtTA$^+$ mice). An inflammatory cardiomyopathy developed in both lines generated (6-mCAR$^+$/αMtTA$^+$ and 12-mCAR$^+$/αMtTA$^+$) following withdrawal of doxycycline. Cardiac CAR was upregulated at 4 weeks of age in 6-mCAR$^+$/αMtTA$^+$ mice and induced a mild inflammatory infiltrate (score 1.3 of 4.0 ± 0.3) at 6 weeks, with 95% of mice surviving to that time. In the second line, 12-mCAR$^+$/αMtTA$^+$ mice, CAR was upregulated in the majority of mice by 3 weeks of age, and by 5 weeks of age more severe cardiac inflammation (score 2.8 of 4.0 ± 0.4) developed with only 56% of mice surviving. The cardiac inflammatory infiltrate was primarily natural killer cells and macrophages in both mCAR$^+$/αMtTA$^+$ lines. A proinflammatory cytokine response with increased cardiac interferon-γ, interleukin (IL)-12, IL-1β, tumor necrosis factor-α and IL-6 was detected by real-time RT-PCR. CAR has been linked to signaling via the inflammatory mitogen-activated protein kinase (MAPK) cascades; therefore, we evaluated the response of these pathways in hearts with upregulated CAR. Both stress-activated JNK and p38MAPK were activated in mCAR$^+$/αMtTA$^+$ hearts prior to onset of inflammation and in isolated mCAR$^+$/αMtTA$^+$ cardiomyocytes. In conclusion, we show for the first time that CAR upregulation in the adult mouse heart induces cardiac inflammation reminiscent of early viral myocarditis. CAR-induced stress-activated MAPK signaling may contribute to the development of cardiac inflammation unrelated to viral infection per se.

1. Introduction

The coxsackie–adenovirus receptor (CAR) is the primary cellular receptor for Group B coxsackieviruses (CVBs) and required for the development of CVB myocarditis [1–5]. CAR is also a high-affinity receptor for adenoviruses, mediating viral attachment [6,7]. In animal models of myocardial inflammation and injury cardiac CAR expression is increased, unrelated to viral infection. It is unclear if induction of cardiac CAR expression during the inflammatory phase of experimental autoimmune myocarditis [8], following myocardial infarction in rats [9] or in human cardiomyopathies is merely the result of myocardial disease. Alternatively, these associations may suggest an active role for CAR in induction of myocardial inflammation.

CAR is recognized to participate in cellular adhesion and intercellular communication, localizing to cell–cell junctions [4,10]. This integral membrane protein is composed of an extracellular region with two immunoglobulin (Ig)-like domains, a transmembrane region and a cytoplasmic domain [1,11]. The intracellular domain has several tyrosine phosphorylation sites with potential to interact with cellular kinases [1,11,12], which may allow interaction with signal transduction pathways [13]. Mitogen-activated protein kinase (MAPK) activation has been repeatedly linked with CAR and CVB and adenoviral infection [14–17]. MAPK activity is regulated by an upstream cytoplasmic cascade of MAPKKKs and MAPKKs. Downstream MAPKs act as signaling

Abbreviations: CAR, coxsackie–adenovirus receptor; CVB, Group B coxsackieviruses; dox, doxycycline; IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; MAPK, mitogen-activated protein kinase; P-MAPK, phosphorylated MAPK; MAPKKK, mitogen-activated protein kinase kinase kinases; MAPKK, mitogen-activated protein kinase kinases; ERK1/2, extracellular-regulated kinases 1 and 2; JNK, c-Jun NH2-terminal kinase; mCAR, murine CAR; αMtTA, α-myosin heavy chain; NK cell, natural killer cell; tTA, tetracycline transactivator; β-gal, β-galactosidase; CSA, cardiomyocyte cross-sectional area; DAPI, 4′,6′-diamidino-2-phenylindole; Ab, antibody; PBS, phosphate-buffered saline; PBST, PBS with 0.1% Tween-20; AEC, aminoethyl carbazole; MCP-1, macrophage chemoattractant protein-1; ECL, enhanced chemiluminescence; HW:BW, heart-to-body weight ratios.

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intermediaries, modulating gene transcription following translocation to the nucleus. Classically, the ERK1/2 pathway is considered to regulate cell growth, proliferation, and differentiation, while JNK and p38 MAPK are thought of as stress-activated MAPKs. All respond to a wide variety of physical, chemical, metabolic, and biologic stimuli, including viral infection [18–20]. In models of CVB myocarditis, ERK1/2 and p38MAPK promote viral infection and inflammation [14,16]. Also, JNK was activated by CVB infection in cell culture, but was not required for propagation of virus [21]. These observations are consistent with a pathway whereby CAR may signal via MAPK activation.

While CAR has been implicated in heart failure, is important to cardiac development [22–26] and has intriguing ties to the immune response, it is not known if the receptor plays an active role in promoting cardiac inflammation. We have previously explored the role of CAR during cardiac development by generating a transgenic mouse model that overexpresses murine CAR (mCAR) in the cardiomyocyte under the control of a tetracycline-responsive α-myosin heavy chain (αMtTA) promoter [26]. Upregulation of cardiac CAR during embryonic development disrupted cardiomyocyte junctions and caused a fatal cardiomyopathy early in life.

To test the hypothesis that CAR activates proinflammatory signals in the adult heart we took advantage of the mCAR$^+$/αMtTA$^+$ model by temporally controlling cardiac expression to delay upregulation to the postnatal mouse. We found that an inflammatory cardiomyopathy developed in association with MAPK activation and increased proinflammatory cytokine expression. Independent of viral infection, the inflammatory cardiomyopathy of mCAR$^+$/αMtTA$^+$ mice mimicked that seen early in viral myocarditis, with natural killer (NK) cell and macrophage infiltration. CAR signaling may therefore influence the pathogenesis of viral heart disease beyond its role as a viral receptor.

2. Methods

2.1. Generation of conditional cardiomyocyte-targeted mCAR1 overexpressing mice

A full-length murine CAR1 (mCAR1) cDNA, tagged at the C-terminus with FLAG was cloned into pBiGN to create pBiGN:mCAR1-FLAG [26,27]. The FLAG tag was incorporated at the C-terminus of mCAR1 to distinguish the transgene product from native mCAR1. Previously we have reported that single-cell pronuclei injections with pBiGN:mCAR1-FLAG produced two pBiGN:mCAR1-FLAG founders with documented germline transmission and CAR transgene expression when bred with α-myosin heavy chain (αMHC) promoter-driven tTA mice (αMtTA$^+$ mice) [26]. Line 6 and line 12 refer to the arbitrary number assigned to these initial founders. The transgene copy number was estimated by Southern blot at 10 to 20 in the 12-CAR line and was approximately half that in the 6-CAR line. Both 6 and 12 lines were backcrossed into a C57BL/6 background, and bred with αMtTA$^+$ mice, also in a C57BL/6 background, to generate CAR$^+$/αMtTA$^+$ strains, capable of conditional cardiomyocyte expression of mCAR1-FLAG. After crossing with αMtTA$^+$ mice we observed CAR upregulation in the developing heart in association with the development of a cardiomyopathic phenotype by 1 week of age in the 12-CAR line [26]. In the absence of doxycycline (dox), the tetracycline transactivator (tTA) binds specifically to pB_{i-1}, the bidirectional promoter of pBIGN, inducing expression of both mCAR1-FLAG and β-galactosidase (β-gal), in this dox-off system. Prior to generating mCAR$^+$/αMtTA$^+$ mice, expression and function of the transgene were confirmed in tTA-expressing CHO-AA8 Tet-off cells [7,28] transiently transfected with pBiGN:mCAR1-FLAG. CVB3 infection confirmed biological activity of the mCAR1-FLAG construct (Supplementary Fig. 1).

To investigate the effect of CAR upregulation in the adult mouse, we took advantage of both CAR lines of mice, crossing each with αMtTA$^+$ mice, to produce 2 conditional lines termed: 6-mCAR$^+$/αMtTA$^+$ and 12-mCAR$^+$/αMtTA$^+$ (and respective 6 or 12 control genotypes: mCAR$^-$/αMtTA$^-$, mCAR$^+$/αMtTA$^-$, and mCAR$^-$/αMtTA$^+$). To suppress mCAR-FLAG expression during development, mice were treated with dox from conception to 1 week of age. Dox (0.2 mg/ml 2.5% sucrose) was administered in drinking water. Dox suppressed mCAR-FLAG expression in postnatal mice when administered to pregnant dams, as we have shown previously [26]. All animal experiments were conducted according to the Hospital for Sick Children and the Toronto Centre for Phenogenomics animal care guidelines.

2.2. Neonatal cardiomyocyte isolation

Neonatal mouse cardiomyocytes were isolated from 3-day-old 6-mCAR$^+$/αMtTA$^+$ mice and cultured as previously described [14,29]. Briefly, hearts were removed, trimmed and minced, then subjected to stepwise enzymatic digestion with 0.15% trypsin (Gibco-BRL, Burlington, ON). Isolated cells were washed with DMEM/HAM F12 [1:1] pH 7.4 with 0.5% penicillin/streptomycin (DMEM/F12) plus 10% FCS (all supplied by Wisent, St. Bruno, QC). Nonmyocytes were depleted by preplating for 60 min at 37°C, then cardiomyocytes were resuspended in media plus 10% FCS, with 0.1 mM 5-bromo-2-deoxyuridine (BRDU; Sigma) (to inhibit growth of nonmyocytes in the presence of serum) and plated in laminin-coated tissue culture plates (Biocoat, Becton-Dickinson, Bedford, MA). After 48 hours adherent cardiomyocytes were washed and resuspended in serum-free DMEM/F-12 plus 5 μg/ml transferrin, 1 nM lithium chloride, 1 nM selenium oxide and 25 μg/ml ascorbic acid. Twenty-four hours later numbers and viability of cardiomyocytes were determined by morphology and contractility, and found to be consistently at a minimum of 85% of adherent cells. In the absence of dox (transgene on) during fetal development and following cardiomyocyte isolation and culture for 72 hours, expression of the CAR-FLAG transgene was confirmed by Western blot (data not shown).

2.3. Histopathology

Cardiac tissue was fixed in 10% formalin, embedded in paraffin and 5 μM transverse cardiac sections were stained with hematoxylin and eosin. Cellular infiltration of the myocardium and cardiomyocyte injury were examined by light microscopy. Severity of myocarditis was scored based on a scale from 0 to 4, as previously described [30]. A score of 0 describes the absence of cellular infiltration or necrosis, 1 represents limited area of inflammation or necrosis, 2 indicates mild to moderate inflammation or necrosis, 3 represents moderate to severe inflammation or necrosis, and 4 describes inflammation or necrosis involving the whole heart [31]. Myocardial inflammation and injury were scored individually by two authors (S.Y. and M.A.O.) in a blinded fashion. Cardiomyocyte cross-sectional area (CSA) was measured at the nucleus level using NIH Image 1.63. One hundred to two hundred cells per mouse were measured, and area was expressed as arbitrary units^2. Picrosirius red staining for collagen deposition and Masson's trichrome stain for fibrosis were performed as previously described [26].

2.4. TUNEL assay

TUNEL staining on 7 μM transverse cryosections of OCT-embedded hearts was performed using Apotag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon International, CA) according to the commercial instructions. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). The percentage of TUNEL-positive nuclei merged with DAPI stained nuclei was determined by counting approximately 2000 DAPI stained nuclei using fluorescence microscopy at ×200 magnification [32].