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Original Article

Substance P is required for the pathogenesis of EMCV infection in mice

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Abstract: Myocarditis is an important cause of heart failure in adolescents and young adults and is caused, most commonly, by viral infections. Viral myocarditis is characterized by cardiac inflammation and cardiomyocyte necrosis. The molecular pathogenesis of viral myocarditis is incomplete and specific therapies are not available. Proinflammatory cytokines such as IL-1β, TNF-α and IL-6 have been implicated in the pathogenesis of myocarditis caused by encephalomyocarditis virus (EMCV) infection, a model of viral myocarditis in mice. Substance P (SP), a neuropeptide and pain transmitter, stimulates the production of proinflammatory cytokines and has been demonstrated by us and others to contribute to the pathogenesis of several viral, protozoan and helminth infections in mouse and man. Receptors for SP are expressed on the surface of cardiomyocytes, neurons, endothelial cells and immunocytes, including lymphocytes and macrophages. The current studies were performed to evaluate the role of SP in the pathogenesis of EMCV-induce myocarditis. SP levels were increased 61 fold in EMCV infected wild-type mice. EMCV infection resulted in 51% mortality at 14 days and a 1.56 fold increase in heart-to-body weight ratio that was accompanied by cardiac inflammation and necrosis and along with cardiomyocyte apoptosis and hypertrophy of surviving cells. In contrast, SP precursor knockout mice were completely protected from EMCV-mortality, cardiomegaly, cardiac inflammation and necrosis as well as cardiomyocyte apoptosis and hypertrophy. These results indicate that SP is essential for the pathogenesis of EMCV myocarditis and suggest that targeting this signaling pathway may be beneficial in viral myocarditis in humans.

Key words: Substance-P, myocarditis, pathogenesis, encephalomyocarditis-virus

Introduction

Myocarditis is an inflammatory disorder of the heart that causes degeneration of the myocardium and is an important cause of heart failure among adolescents and young adults [1]. Myocarditis is caused most commonly in developed countries by viral infections such as coxsackie virus, echovirus, adenovirus and picornovirus; myocarditis in developing countries occurs more commonly as a complication of bacterial or parasitic infections, most notably *Streptocoocus pyogenes* and *Trypanozoma cruzi*, respectively [2-6]. There is currently no specific treatment for viral myocarditis [7-10].

Murine myocarditis caused by infection with encephalomyocarditis virus (EMCV) is a commonly used experimental model to study viral myocarditis [11-15]. EMCV infection in mice is a fatal disease accompanied by increased heart weight, cardiac inflammation and necrosis, cardiomyocyte apoptosis and hypertrophy with resultant myocardial damage and cardiac failure [11, 16-20]. The molecular basis for these changes have not been delineated, although proinflammatory

cytokines such as IL-1β, TNF-α and IL-6 have been implicated in the pathogenesis of myocardial injury in viral myocarditis [16, 21, 22].

Substance P (SP), a neuropeptide and pain transmitter, is known to stimulate production of proinflammatory cytokines and to stimulate proinflammatory cell chemotaxis [23-33]. Receptors for SP are expressed on the surface of cardiomyocytes as well as neurons, endothelial cells and immunocytes, such as lymphocytes and macrophages [34-37]. Furthermore, we previously demonstrated that SP contributes to a dilated cardiomyopathy, which accompanies *Taenia crassiceps* infection in mice [38].

The current studies were performed in order to evaluate the role of SP in the pathogenesis of EMCV-induced myocarditis. We compared mortality, heart-to-body weight ratio, cardiac inflammation and necrosis and cardiomyocyte apoptosis and cell diameter in EMCV infected wild type and substance P precursor knockout mice. SP precursor knockout mice were completely protected from EMCV-mortality, cardiomegaly, cardiac inflammation and necrosis as well as cardiomyocyte apoptosis and hypertrophy. These results indicate that SP is essential for the pathogenesis of EMCV myocarditis and suggest that targeting this signaling pathway may be beneficial in viral myocarditis in humans.

Materials and methods

EMCV model of murine myocarditis

All of the animal procedures were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine and were performed in compliance with the National Institutes of Health regulations for animal handling and usage. C57BL/6 mice were purchased from Harlan-Sprague; SP precursor knockout mice backcrossed for >10 generations into the C57BL/6 background were originally purchased from Jackson laboratories; a breeding colony was maintained at Baylor College of Medicine in a pathogen-free mouse facility. The EMCV model of murine myocarditis used in these studies was as previously described [16]. A myocarditic variant of EMCV that was generously provided by Dr. Sally Huber (University of Vermont) was used for these studies. The virus stock was stored at −80°C in Hanks' balanced salt solution with 0.1% BSA until use. Fifty plaque-forming units (pfu) were injected intraperitoneally into 8-week old male wild type or SP precursor knockout mice. Mice were housed in a biohazard facility and observed twice daily for the duration of the experiments. Mice that became moribund were considered to have reached the end point of the experiment and were euthanized. Mice were sacrificed at 14 days post-infection and their hearts were removed and heart-to-body weight ratio, cardiomyocyte diameter determination and histopathological examination performed as outlined below.

Quantitation of SP protein

Extraction and quantitation of SP protein was performed as described previously [39]. Briefly, heart was homogenized in 1% trifluoroacetic acid (TFA; 1ml/gram of tissue) and centrifuged at 17,000 g for 15 minutes at 2-8^0 C. The supernatant was then passed through a Sep-Pak C18 Cartridge (Waters, Associates, Milford, MA). The cartridge was washed with 10-20 ml of 1 % TFA and the protein eluted with 3 ml of a 60:40 solution of acetonitrile: 1% TFA. SP protein in the eluants was quantitated using ELISA kit (cat no. DE1400, R&D systems, Minneapolis, MN). Total protein was quantitated using the Bradford method (cat no. 500-0006, Bio-Rad, Hercules, CA). Results are expressed as picograms of SP per mg of total protein.

Measurement of myocardial heart-to-body weight ratio

Mice were weighed and anesthetized followed by sacrificing by cervical dislocation. The chest cavity was rapidly opened, and the heart removed and weighed. The heart weight relative to body weight was calculated.

Histopathological study

Hearts were fixed in 4 % paraformaldehyde and embedded in paraffin. Heart sections were stained with hematoxylin and eosin and examined microscopically at 200X magnification. The inflammatory score was determined as described by Kanda et al. [40] and was as follows: 0, no lesions; 1+, lesions involving <25% of the ventricular myocardium; 2+,lesions involving 25–50% of the myocardium; 3+, lesions involving 50–75% of

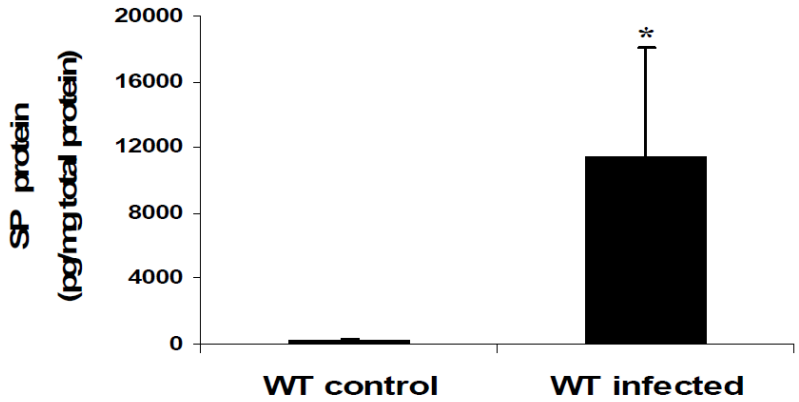

Figure 1. Effect of EMCV infection on substance P protein levels in wild type mice. SP levels were determined in control uninfected (n=5) and EMCV infected wild type mice (n=3). Data presented are mean ± SD (*,SP levels in control uninfected versus EMCV infected wild type mice, p = 0.05, Student's t-test).

the myocardium; and 4+ lesions involving 75–100% of the myocardium. The extent of cardiac necrosis was graded blindly by an experienced histopathologist who had no knowledge of the study design and was based on the percent of ventricular mural myocardium affected, or showing signs of necrosis. This grading was based upon the percentage of thickness of ventricular wall affected (100% being full-thickness). Cardiac hypertrophy was determined by measuring mean cardiomyocyte diameter as determined by measuring the diameter of 50 myocytes using NIH IMAGE v.1.62 software.

TUNEL assay

The ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon) was used to detect apoptotic cardiomyocytes and was performed according to the manufacturer's instructions, as described previously [38]. Heart tissue sections were deparaffinized and rehydrated by washing sections in 3 changes of xylene, 2 changes of ethanol, one change in 95% ethanol, one in 70% ethanol and one in 1X PBS (5 min each wash). The heart tissues were then treated with proteinase K (20µg/ ml, 15mins, RT), washed twice with distilled water (2 min each wash), quenched with 3.0% hydrogen peroxide in PBS (Sigma, 30 min, RT), followed by two washes with PBS (5 min each rinse), treatment with equilibration buffer (10 sec, RT) and TdT enzyme (37^{0} C for 1 hr). The tissue sections were then treated with stop-wash buffer, and washed with PBS 3 times (1 min each wash) followed by treatment with anti-digoxigenin conjugate (30 min, RT), 1 wash with PBS (1 min, RT) and treatment with peroxidase substrate (3–6 min, RT) and 3 washes with distilled water (1 min each), counterstained with hematoxylin, mounted with permount, and viewed under a light microscope. The number of TUNEL-positive nuclei was counted in five-to-ten randomly chosen high-power fields (1000x) of each slide by an experienced microscopist blinded to the study design. The slides were graded on a scale of 1+ to 4+ as follows, 0, no apoptotic cells; 1+, <25% of the cardiomyocytes are apoptotic, 2+, 25–50% of the cardiomyocytes are apoptotic; 3+, 50–75% of the cardiomyocytes are apoptotic; and 4+, 75–100% of the cardiomyocytes are apoptotic. Data presented are mean apoptotic score ± SD for each group; significant differences are indicated (using Student-t-test).

Statistical analysis

Statistical analysis was done using Primer software (Stanton A. Glantz, 1992, McGraw-Hill, Ohio). Statistical differences were determined using Student'ts t-test or the Kaplan-Meier analysis as indicated. Significance was set at $P<0.05$.

Results

Effect of EMCV infection on heart SP protein levels

To begin to assess the contribution of SP to EMCV-induced myocarditis, we measured SP protein levels within the hearts of wild type C57BL/6 mice 14 days after infection. SP protein within hearts of EMCV-infected wild type mice (11,422 ± 6700 pg/mg total protein; **Figure 1**) was increased 61 fold compared to hearts from uninfected, age-matched mice (186 ± 113 pg/mg total protein; p=0.05, Student's t-test).

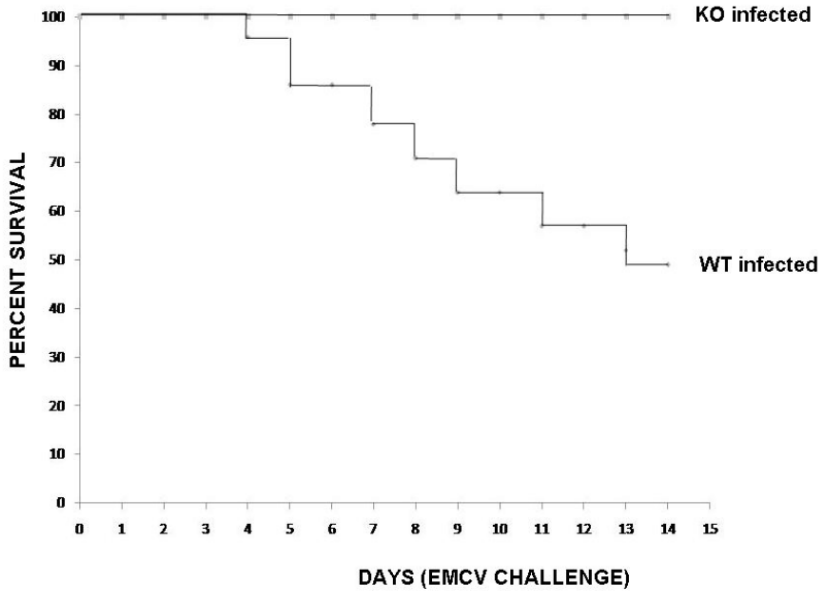

Figure 2. Effect of EMCV infection on mortality in wild type mice vs. SP precursor knockout mice. Percent survival rate was determined in EMCV infected wild type mice (n=27) and SP precursor knockout mice (n=11) over 14 days following infection (p=0.005, Kaplan-Meier analysis).

Effect of EMCV infection on mortality and heart size in wild type versus SP precursor knockout mice

The SP precursor knockout mouse was bred into the C57BL/6 background. EMCV infection has been examined in several mouse strains [11, 16-20, 41-44], but studies using the C57BL/6 strain are limited. Existing studies have used a EMCV dose ranging from 10-500pfu's in C57BL/6 mice [12, 42-46]. These studies have demonstrated that C57BL/6 mice are susceptible to an EMCV dose as low as 10 pfu with a mortality rate of 85% on day 14 following infection [45]. Other studies demonstrated a mortality rate of 5-58 % on day 14 with 500 pfu of the EMCV virus [12, 42, 46]. A study by Wada et al, showed a mortality rate of 5% on day 14 in C57BL/6 mice infected with 50 pfu of EMCV [46].

We demonstrated a mortality rate of 51% at day 14 in C57BL/6 wild type mice following infection with 50 pfu of the EMCV virus (**Figure 2**). Most importantly, no deaths were observed in the EMCV infected SP precursor knockout C57BL/6 mice ($p< 0.05$, Kaplan-Meier analysis; **Figure 2**).

Studies on heart-to-body weight ratios following EMCV in C57BL/6 are limited. Most studies in C57BL/6 mice have examined the effects of EMCV infection on heart-to-body weight ratio in comparison with other knockout mice and have not reported differences in heart-to-body weight ratio in infected vs. uninfected wild type mice [42-44, 47], although one study by Yamamoto et al, reported that compared to uninfected/PBS treated mice, there was a 11% increase in the heart-to-body weight following infection of 10 pfu of EMCV in C57BL/6 mice [45].

Our results demonstrated that the mean heart-to-body weight ratio following infection of 50 pfu of EMCV in C57BL/6 mice (0.0111) was 56% higher compared to that of uninfected mice (0.0071, $p< 0.001$, Student's unpaired t-test; **Figure 3A**). Importantly, there was no statistically significant difference in the heart-to-body weight ratio between infected and uninfected SP precursor KO mice (**Figure 3A**). Also, the mean heart-to-body weight ratio in the infected C57BL/6 wild type mice (0.0111) was 76% higher that that of the infected SP precursor knockout C57BL/6 mice (0.0063, $p< 0.001$, Student's unpaired t-test; **Figure 3A**). In addition, the hearts of EMCV infected WT mice were enlarged compared to hearts of the uninfected WT mice and the hearts of the infected or the uninfected SP precursor knockout mice (**Figure 3**, **B**, **D**, and **E**).

Effect of EMCV infection on cardiac inflammation and necrosis and cardiomyocyte apoptosis and hypertrophy in wild type versus SP precursor knockout mice

Histopathological changes, including cardio-myocyte hypertrophy, cardiac inflammation

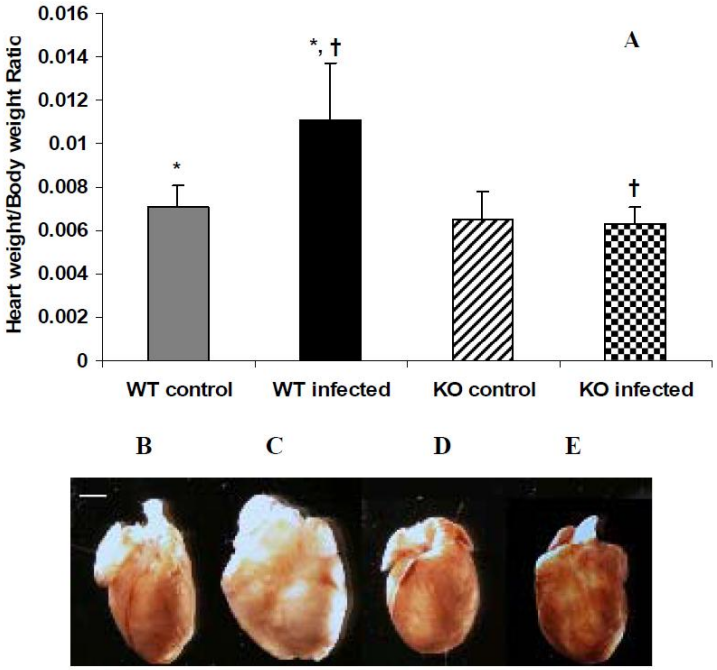

Figure 3. Effect of EMCV infection on heart size in wild type and SP precursor knockout mice. *Upper panel*: Heart-to-body weight ratios of EMCV uninfected and infected WT and SP precursor knockout mice (n=8-13). Results are expressed as the mean ± SD of 2 separate experiments (*, Heart-to-body weight ratios of EMCV uninfected versus infected mice, p< 0.001, Student's unpaired t-test)(†, Heart-to-body weight ratios of EMCV infected, WT versus SP precursor knockout mice, p< 0.001, Student's unpaired t-test). *Lower panel*: Gross appearance of a representative heart from A) uninfected wild type mouse, B) EMCV-infected wild type mouse, C) uninfected SP precursor knockout mouse and D) EMCV-infected SP precursor knockout mice (white bar = 1mm).

and necrosis and cardiomyocyte apoptosis following EMCV infection have been examined in several mouse strains [11, 16-20, 41-44, 45], but studies using the C57BL/6 strain are limited. Our results demonstrated a cardiac inflammatory influx only in the EMCV infected C57BL/6 wild type mice but not in infected SP precursor knockout C57BL/6 mice (**Figure 4**). The average inflammatory score in the EMCV infected C57BL/6 wild type mice was 1.5 ± 0.55 compared to none in the infected SP precursor knockout C57BL/6 mice (p< 0.001, Student's t-test; **Figure 4E**).

Previous studies have reported that 25-75% of the myocardium demonstrated necrosis in response to EMCV infection in C57BL/6 mice [41, 42, 44, 45]. We confirmed these findings by demonstrating that 60±20% of the ventricular mural myocardium of EMCV infected C57BL/6 wild type mice demonstrated necrosis. In contrast, necrosis was not observed in EMCV infected SP precursor knockout C57BL/6 mice (p< 0.001, Student's t-test; **Figure 4F**).

We also assessed hearts of EMCV-infected mice for cardiomyocyte apoptosis. Cardiomyocyte apoptosis was observed only in the EMCV-infected C57BL/6 wild type mice and not in the infected SP precursor C57BL/6 mice or either of the uninfected control groups (**Figure 5**). The average apoptosis score in the infected C57BL/6 wild type mice was 1.33 ± 0.58. In contrast the SP precursor KO C57BL/6 mice did not demonstrate any cardiomyocyte apoptosis (p= 0.0080, Student's t-test; **Figure 5E**). Thus, SP is required for cardiac, inflammation, necrosis and apoptosis in mice following EMCV infection.

Previous studies reported a 25-30% increase in the diameter of cardiomyocytes in DBA/2 mice following EMCV infection [11, 16-20][41]. Cardiomyocyte diameter determinations following EMCV infection in C57BL/6 mice have not been reported. The mean cardiomyocyte diameter was 12.37±0.71 μm in the infected C57BL/6 wild type mice versus 10.6±0.64 μm in the uninfected mice (p<0.001, Student's t-test; **Figure 5**). There was no significant difference in the cardiomyocyte diameter in infected versus uninfected SP precursor KO C57BL/6 mice (**Figure 5**).

Discussion

The current studies were performed in order to evaluate the role of SP in the pathogenesis of EMCV myocarditis. We demonstrated that SP levels were increased in EMCV infected wild type C57BL/6 mice. The increased cardiac SP levels in response to EMCV infection in the wild type C57BL/6 mice was accompanied by increased mortality, heart-to-body weight ratios and histopathological changes including

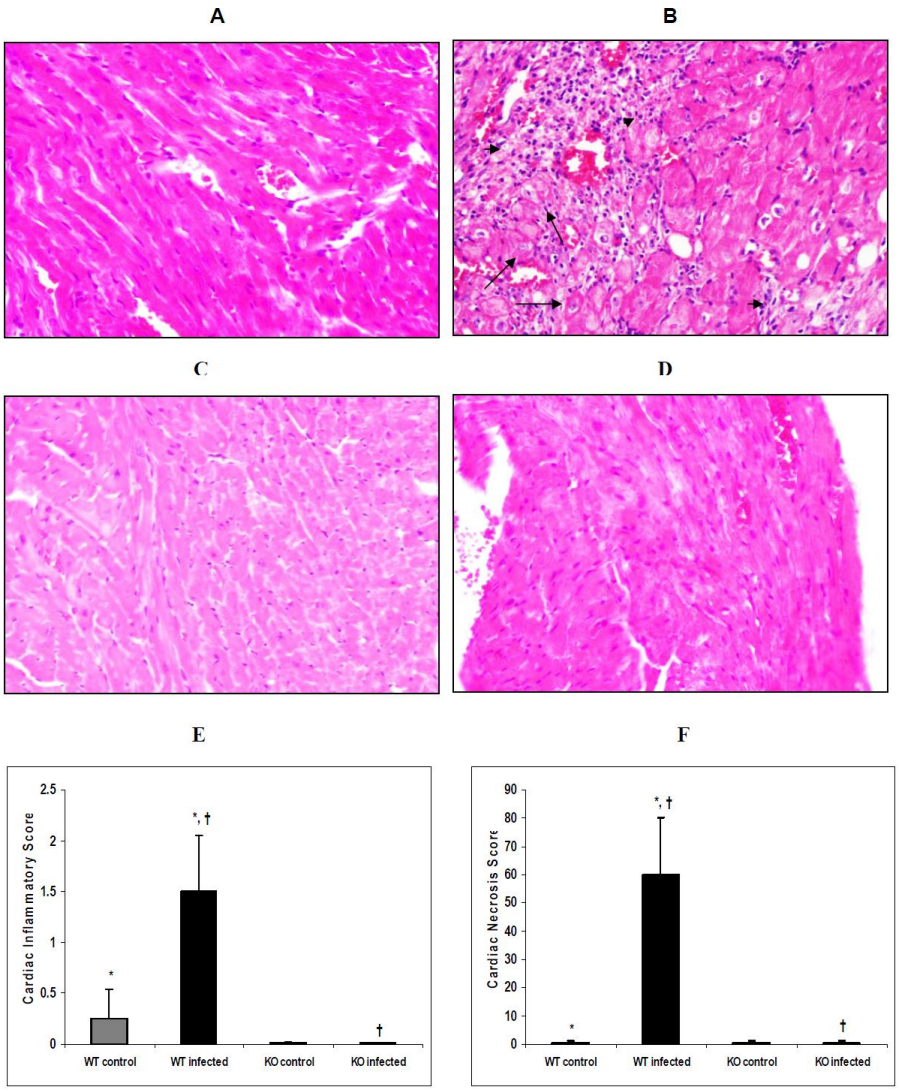

Figure 4. Effect of EMCV infection on cardiomyocyte inflammation and necrosis in wild type and SP precursor knockout mice. Paraffin-embedded heart sections were deparaffinized and stained with hematoxylin and eosin and were graded for cardiac inflammation and necrosis. Photomicrographs of an H&E stained section of heart from (A) an uninfected wild type mouse showing no inflammatory influx, (B) a EMCV-infected wild type mouse showing numerous infiltrating cells (arrowheads pointing to inflammatory cells and arrows point to necrotic cells) (C) an uninfected SP precursor knockout mice showing no inflammatory influx and (D) a EMCV-infected SP precursor knockout mice showing no inflammatory cells (original magnification 1000 x). Cardiac inflammatory score (E) and cardiac necrotic score (F) of EMCV infected, wild type (n= 6) and SP precursor knockout mice (n= 4). Results are expressed as the mean ± SD of 2 separate experiments (∗, Cardiac inflammatory and necrosis score of EMCV uninfected versus infected mice, $p< 0.001$, Student's unpaired t-test)(†, Cardiac inflammatory and necrosis score of EMCV infected, WT versus SP precursor knockout mice, $p< 0.001$, Student's unpaired t-test).

cardiac inflammation and necrosis, cardiomyocyte apoptosis and hypertrophy. Remarkably, all of the manifestations of EMCV infection were absent in SP precursor knockout C57BL/6 mice indicating that SP is required for the pathogenesis of EMCV myocarditis.

The mechanism leading to heart enlargement in the EMCV infected wild type mice may be due to the inflammatory influx and cardiomyocyte hypertrophy. Our results demonstrated that heart enlargement does not occur in the absence of SP in the SP

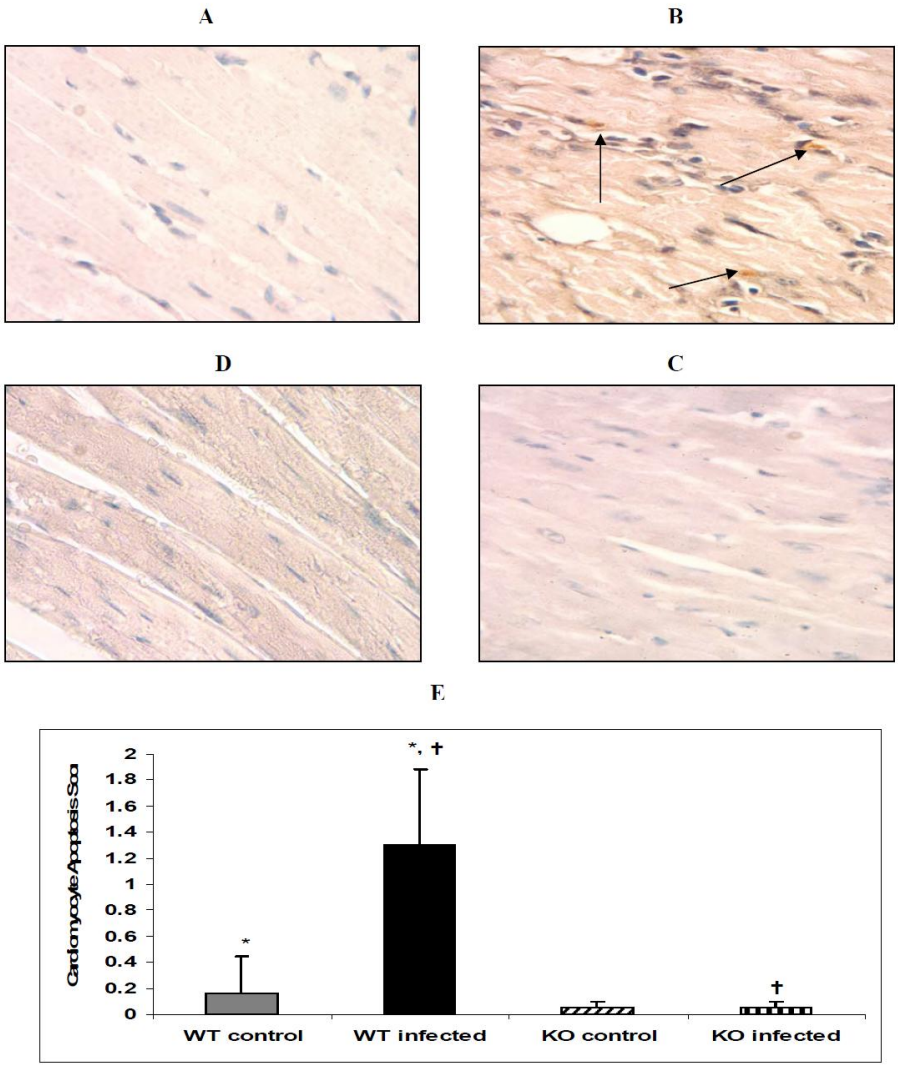

Figure 5. Effect of EMCV infection on cardiomyocyte apoptosis in wild type and SP precursor knockout mice. TUNEL staining of heart sections from (A) an uninfected wild type mouse showing no TUNEL-positive nuclei, (B) an EMCV-infected wild type mouse (arrows point to TUNEL-positive cardiomyocytes), (C) an uninfected SP precursor knockout mice showing no TUNEL-positive nuclei and (D) an EMCV-infected SP precursor knockout mice showing no TUNEL-positive nuclei (original magnification 1000 x). Panel E shows cardiomyocyte apoptotic scores of EMCV-infected C57BL/6 wild type mice (n= 6) and SP precursor knockout mice (n= 4). Results are expressed as the mean ± SD of 2 separate experiments (∗, Cardiomyocyte apoptosis score of EMCV uninfected versus infected mice, $p< 0.05$, Student's unpaired t-test)(†, Cardiomyocyte apoptosis score of EMCV infected, WT versus SP precursor knockout mice, $p< 0.01$, Student's unpaired t-test).

precursor knockout mice. Substance P (SP) is known to specifically stimulate the chemotaxis of proinflammatory cells and is also known to stimulate production of proinflammatory cytokines [23-33]. Thus our results demonstrating that in the absence of SP, inflammatory influx does not occur in the EMCV-infected SP precursor knockout mice, implicates that SP is one of the key mediators involved in the cardiac inflammatory responses associated with EMCV myocarditis.

The current results also demonstrated that cardiac necrosis and apoptosis occurs in response to EMCV infection in wild type mice but not in SP precursor knockout mice. Cardiac necrosis and apoptosis may result from SP-induced mediators such as

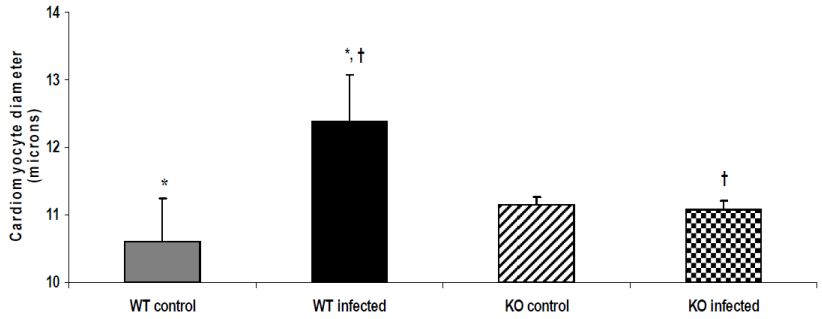

Figure 6. Effect of EMCV infection on cardiomyocyte diameter in wild type and SP precursor knockout mice. Cardiomyocyte diameter (μm) at the nucleus was determined in hearts from wild type uninfected (n=4), wild type infected (n=3), SP knockout uninfected (n=3), and SP knockout infected mice (n=3). Fifty cardiomyocytes were counted in each heart using NIH IMAGE v.1.62 software. Results are expressed as the mean ± SD of 2 separate experiments (∗,Cardiomyocyte diameter of EMCV uninfected versus infected mice, p< 0.001, Student's unpaired t-test)(†, Cardiomyocyte diameter of EMCV infected, WT versus SP precursor knockout mice, p< 0.001, Student's unpaired t-test).

proinflammatory cytokines. Proinflammatory cytokines such as TNF-α, IL-6 and IL-1β have been implicated in the pathogenesis of myocardial injury in viral myocarditis [16, 21, 22]. In the murine model of EMCV myocarditis, TNF-α is known to be elevated in the acute stage and exogenously administered anti-TNF-α antibody improved survival and reduced the myocardial injury [21]. Overexpression of IL-6, another pro-inflammatory cytokine is also known to modify viral myocarditis. After inoculation with EMCV, the heart-to-body weight ratio and myocardial injury were significantly increased in IL-6 transgenic mice [48]. IL-1β gene expression is high in the chronic stage of EMCV myocarditis and correlated with increased heart-to-body weight ratio and with the extent of fibrotic lesions [49]. Interaction of SP with its receptor is known to activate RhoA, a small G-protein. RhoA activation has been shown to induce hypertrophy and apoptosis of cardiomyocytes in vitro [50].

EMCV infection in mice has been shown to lead to cardiac remodeling [11, 14]. However the mechanisms involved in the EMCV-associated cardiac remodeling are unknown. Other studies have shown that inflammatory mediators like TNF-α may play an important role in cardiac remodeling, including cardiomyocyte hypertrophy, alterations in fetal gene expression, and progressive cardiomyo-cyte loss through apoptosis [51, 52, 53]. In transgenic mice with targeted TNF-α over-expression, cardio-myocyte apoptosis has been shown to induce adverse cardiac remode-ling [54]. In our studies, we noted both cardiomyocyte apoptosis and necrosis in wild type mice that are infected with EMCV. We speculate that apoptosis and/or necrosis following EMCV infection in the wild type mice may lead to cardiomyocyte loss that in turn could lead to adverse cardiac remodeling as a consequence of pro-gresssive ventricular wall thinning. Substance P may be responsible for cardiac remodeling in EMCV infection either directly or indirectly via stimulation of proinflammatory cytokines such as TNF-α.

Matsumori et al have shown that several agents have beneficial effects in myocarditis associated with EMCV in animals including amlodipine (a calcium channel blocker), vesnarinone (a positive inotropic agent), TCV-116 (an angiotensin II type I antagonist), ribavirin (a nucleoside analogue), recombinant alpha interferon, carteolol (a nonselective beta-adrenergic blocker with intrinsic sympathomimetic activity) and pycnogenol (an extract of French maritime pine bark) [18-21, 57-59]. However, none of these agents has demonstrated utility in the treatment of patients with viral myocarditis. Our finding that SP is a key mediator of EMCV myocarditis in mice suggests a new approach to the treatment of patients with viral myocarditis. SP mediates its effect by binding to one or more of three receptors—the neurokinin-1 receptor (NK1R), NK2R or NK3R. Currently, the NK1R antagonist, aprepitant (Emend®) is FDA-licensed for treatment of chemotherapy-induced nausea and vomiting. The NK2R antagonist, saredutant (Sanofi-Aventis, Bridgewater, New Jersey) is in Phase III trials for use in depression; the NK3R antagonist, talnetant (GlaxoSmithKline, London, UK) is in

phase II trials for use in schizophrenia. Studies are underway in our laboratory to assess which receptor(s) mediates the SP effect and which antagonist(s), therefore, potentially may be beneficial in treating patients with viral myocarditis.

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