

Selenium deficiency contributes to the chronic myocarditis in coxsackievirus-infected mice

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Received August 8, 2010; accepted January 22, 2011

Summary. – Both coxsackievirus B3 (CVB3) infection and selenium (Se) deficiency play a pivotal role in Keshan disease of the heart. The Se deficiency was known to contribute to the CVB3-induced myocarditis in acute and subacute phase of infection. However, its effect on the myocarditis in chronic phase of infection has not been examined yet. To address this question, we kept mice on a Se-replete or Se-deficient diet for 28 days, infected them intraperitoneally with CVB3 and maintaining previous diets, we examined them for next 90 days for several parameters indicative of the infection or disease. We found out that the mice on the Se-deficient diet exhibited a higher mortality, lower serum glutathione peroxidase (GPx) activity, evident histopathological changes indicative of myocarditis, and a higher level of viral RNA in the heart. Summing up, these data suggest that the Se-deficiency creates a chronic myocarditis-prone condition by fostering the active virus replication.

Keywords: coxsackievirus B3; mice; selenium deficiency; myocarditis; glutathione peroxidase; viral RNA

Introduction

Coxsackievirus B3 belongs to the human enterovirus B group in the genus *Enterovirus* of the family *Picornaviridae* (Fauquet *et al.*, 2005). CVB3 is a non-enveloped virus containing single-stranded positive-sense RNA genome with a single ORF. CVB3 can cause a wide variety of acute human diseases ranging from mild rash or cold-like symptoms to the severe conditions including myocarditis (Pallansch and Roos, 2001; Tracy and Drescher, 2008). In addition, CVB3 has been implicated in some several chronic diseases notably cardiomyopathy (Muir, 1993; Roivainen, 1999).

Selenium is a trace mineral that plays a crucial role in the protection against oxidants, serving as an essential component of a number of antioxidant enzymes including glutathione peroxidase (GPx) and glutathione reductase (Stadtman, 2000). Keshan disease, initially identified in particular areas

of China, has a dual etiology: low dietary intake of Se and CVB infection (Beck *et al.*, 2003, 2007). This disease represents a very serious cardiomyopathy characterized by the extensive necrotic lesions with inflammatory heart infiltrate and calcification (Ge *et al.*, 1983). Genotypic analysis of the blood or tissue samples from Keshan disease patients further suggested that the most common CVB type is CVB3 (Li *et al.*, 2000). Experiments using animal model showed that Se deficiency allowed a non-myocarditic CVB3 to mutate into a virulent myocarditic virus (Beck *et al.*, 1994, 1995).

A mouse is well-established model for studying the pathogenesis of CVB3 infection and experiments with this animal have provided insight into the CVB3-associated human diseases. However, most studies have focused on the role of CVB3 during the acute or subacute phase (Huber and Lodge, 1986; Herskowitz *et al.*, 1987; Joo *et al.*, 2003). However, the study of chronic phase is equally important, since CVB3-induced chronic myocarditis is likely to progress to the dilated cardiomyopathy that can be treated only by heart transplantation. In addition, very limited information is available on the overall pathogenic characteristics of CVB3 infection under the Se-deficient diet during chronic infection phase.

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Abbreviations: CVB3 = coxsackievirus B3; GPx = glutathione peroxidase; p.i. = post-infection; Se = selenium

In this study, we investigated the effect of Se deficiency on a development of myocarditis in CBV3-infected mice, particularly in late phase of infection. The mice were followed for morbidity, mortality, histopathological changes, and viral RNA level in the heart.

Materials and Methods

Cells, virus, and animals. Vero cells from the American Type Culture Collection (ATCC) were maintained in DMEM (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 2 mmol/l L-glutamine, 100 IU/mmol/l penicillin, and 50 µg/ml streptomycin. CVB3 (Nancy strain, VR-30) obtained from ATCC was propagated in Vero cells. Viral stocks were prepared, stored, and titrated as described previously with minor modifications (Moon *et al.*, 2005). Three-week-old male A/J mice (Shizuoka Laboratory Animal Center, Japan) were maintained in a temperature-controlled room.

Mice experiments. Mice were divided into two groups. First group was fed on a diet with adequate amounts of Se (Se-replete diet, n = 87) and the second one on a Se-deficient diet (n = 109). Mice were commenced on the special diet 28 days before virus inoculation and maintained on the same diet for 90 days post-infection (p.i.). Both Se-replete (TD 92198) and Se-deficient feedstuff (TD 92163) were purchased from Harlan Teklad. Both groups of mice were inoculated intraperitoneally with 5×10^5 PFU of CVB3 in the volume 100–200 µl of infected-Vero cell lysate (n = 166). Mice in the control group were inoculated with non-infected Vero cell lysate (n = 30).

Histopathology. Tissues collected by sacrificing mice at the designated times were aseptically separated into two portions. One half of each tissue was rapidly fixed in a 4% (v/v) paraformaldehyde solution (Sigma-Aldrich) to prepare 3 µm-thick paraffin-embedded sections. Sections were stained with hematoxylin/eosin and observed under a light microscope. Histopathologic scores were determined by examination of the staining patterns by 3 independent investigators blinded to the sample identity. Inflammatory myocarditis findings in the heart sections were evaluated on a scale of 0 to 5 according to the percentage of heart section showing inflammation relative to the overall section size as follows: 0 (no inflammation), 1 (1–10%), 2 (11–20%), 3 (21–30%), 4 (31–40%), 5 (>50%).

Glutathione peroxidase activity. Plasma GPx activity was measured using a Glutathione Peroxidase Assay Kit (Cayman Chemical Company) as recommended by the manufacturer. Briefly, oxidized glutathione (GSSG) produced upon the reduction of hydroperoxide by GPx was recycled to the reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADPH⁺ was accompanied by a decrease in A₃₄₀. Under conditions in which GPx activity was rate-limiting, the rate of decrease in A₃₄₀ measured spectrophotometrically, was directly proportional to GPx activity expressed in nmoles/min/ml.

Real-time RT-PCR. RNA was isolated using the TRIzol reagent (Invitrogen) and dissolved in 50 µl of nuclease-free water. The fol-

lowing primer and probe sequences were utilized (ABI): 5'-GCT TTG CAG ACA TCC GTG ATC-3' (forward primer); 5'-CAA GCT GTT CCA CAT AGT CCT TCA-3' (reverse primer); and 5'-(6-FAM) TGT GGC TGG AAG ATG ATG CAA TGG A (TAMRA)-3'. The first strand cDNA was generated using CVB3 reverse primer Superscript reverse transcriptase (Invitrogen). The mixture for PCR contained CVB3 reverse as well as forward primers and fluorogenic probe. PCR consisted of a denaturation step (15 secs at 95°C) and combined annealing-extension step (1 min at 60°C). The amount of product amplified was expressed as a normalized fluorescence (Rn) representing the change in target fluorescence relative to that of a housekeeping gene.

Statistical analysis. Data were expressed as means ± standard deviations or standard errors and as the mean logarithmic values. Differences between groups were analyzed by the chi-squared and Student's *t*-tests using SPSS software (Statistical Package for the Social Sciences, Version 9.05, SPSS Inc., Chicago). A difference of P ≤ 0.05 was considered as statistically significant.

Results

Morbidity and mortality

We split the experimental infection into three phases: acute (1–4 days p.i.), subacute (5–14 days p.i.), and chronic phase (15–90 days p.i.) according to the time after virus inoculation (Reetoo *et al.*, 2000). During the subacute phase, mice in both Se-deficient and Se-replete groups lost their weight and then gradually gained (Fig. 1a). All CVB3-infected mice experienced deterioration in health after the acute/subacute stage exhibiting crooked backbones and paralyzed legs. At day 3 p.i., both groups began to suffer a severe mortality (Fig. 1b). This rapid drop in viability continued till day 7 p.i. before flattening out. The mortality was higher in the group of mice on the Se-deficient diet than on Se-replete diet. The survival rates after CVB3 infection were 37.6% and 52.5% for Se-deficient and Se-replete mice, respectively. In agreement with these data, the median survival of infected mice was 7 days for the Se-deficient group and 90 days in the Se-replete group. Uninfected control mice remained healthy for the duration of experiment regardless of the Se dietary status.

Serum GPx activity

To determine the actual effect of Se deficiency, we quantified GPx activity in serum as a Se-biomarker (Stadtman, 2000). After 28 days of a Se-controlled diet, the mice fed on the Se-deficient diet experienced more than a twofold decrease in GPx activity compared to the animals on the standard diet (P < 0.05; Fig. 2). Enzymatic activity was determined in six mice before the virus inoculation and in two

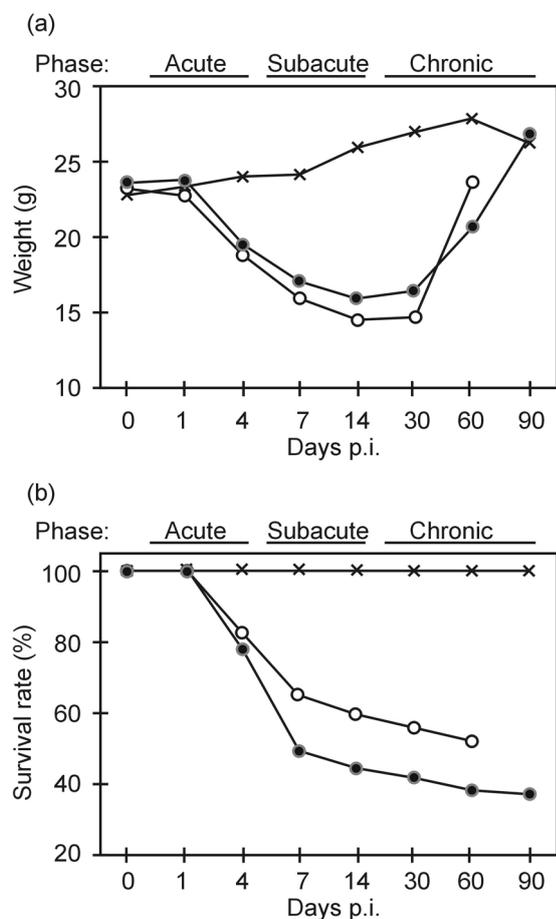


Fig. 1

Effects of the Se diet on the body weight (a) and the survival rate (b) of CVB3-infected mice

Non-infected (x), Se-replete (o), and Se-deficient (•) mice.

mice after the inoculation. Under Se-deficient conditions, GPx activities were consistently and significantly lower than that under Se-replete conditions. In contrast, there was only a slight decrease of GPx activity in the CVB3-infected Se-replete mice. Interestingly, GPx activity on day 1 p.i. was markedly increased in this group ($P < 0.05$). These data thus indicate that the Se concentration *in vivo* decreased significantly in the mice on Se-deficient diet as reflected by the fall of GPx activity. Furthermore, GPx activity declined to the undetectable levels during the chronic phase of CVB3 infection.

Histopathological changes in the heart

Histological changes in the heart tissues were analyzed at the designated time points p.i. Extensive cellular infiltration was observed in both groups of CVB3-infected mice particularly during the subacute phase (Fig. 3 and Table 1).

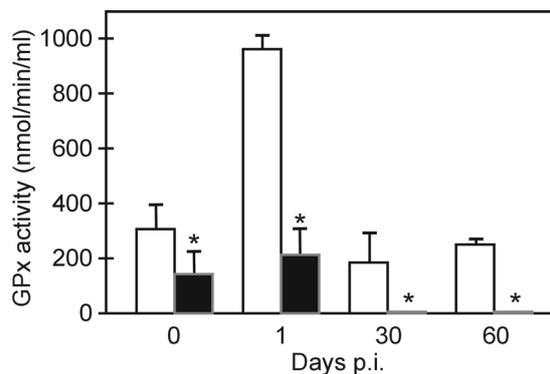


Fig. 2

Effects of the Se diet on the serum GPx activity in CVB3-infected mice. Se-replete (empty columns) and Se deficient (full columns) mice. (*) represents $P < 0.05$.

Inflammatory signals were first detected on day 7 p.i. in both groups and pathological scores were moderate to severe with no obvious difference between groups (Fig. 3 and Table 1). The incidence of myocarditis during the subacute phase was observed in 9 of 10 tissues in the Se-replete group and in 10 of 10 tissues in the Se-deficient group. Severe inflammatory response slowly declined in both groups thereafter. By day 60 p.i., there was no indication of inflammation in the group of Se-replete mice. In contrast, 2 of 9 Se-deficient mice exhibited changes indicating that the inflammation persisted to a certain degree during the chronic period. Taken together, these results implied that chronic myocarditis developed preferentially under Se-deficient conditions following CVB3 infection.

Viral RNA in the heart

The kinetics of virus replication in heart tissue was studied during the course of CVB3 infection by quantifying viral RNA levels using real-time RT-PCR. Viral RNA was detected in both groups of CVB3-infected mice at all time points p.i. Viral mRNA levels peaked 4–7 days p.i., when the average number of viral RNA copies was $7.5 \pm 0.3 \times 10^7$ in both animal groups (Fig. 4). These data indicated that there was no significant difference in the viral RNA level between the two groups of mice during the early stage of infection. During the post-subacute period, the amount of viral RNA dramatically decreased on day 30 p.i. in the group of Se-replete mice. In contrast, at the same time point, the group of Se-deficient mice maintained a much higher level of viral RNA that was comparable to the maximal RNA level observed during the acute phase of infection. A similar pattern of the viral RNA persistence was also evident in the pancreas (data not shown). Taken together, the heart histopathology data suggested that Se deficiency contributed

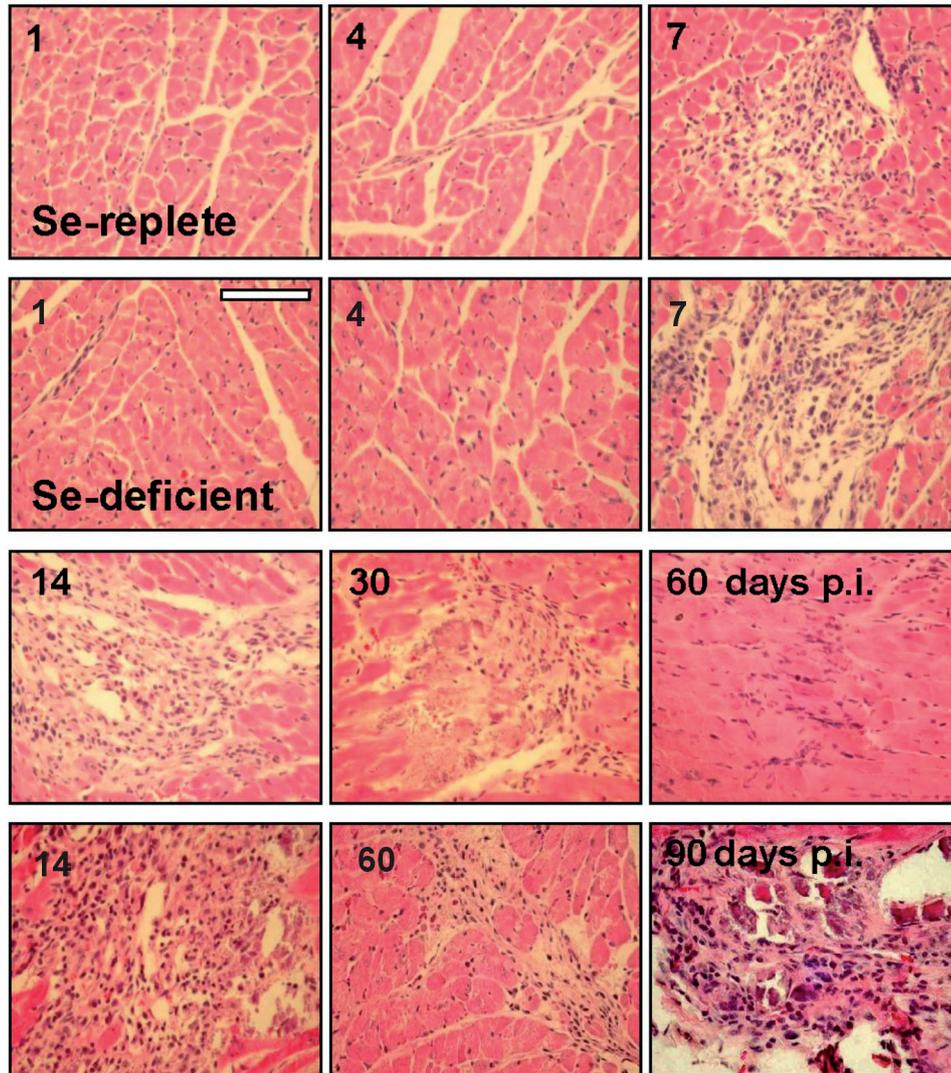


Fig. 3

Effects of Se diet on the histopathological changes in the heart of CVB3-infected mice

At designated days p.i., the heart tissues were stained with hematoxylin and eosin. Magnification 200x, scale bar represents 100 μ m.

Table 1. Effect of Se diet on the development of myocarditis in CVB3-infected mice determined by histopathology

Condition	Pathological grades	Number of mice with assessed grade					
		Acute phase/days		Subacute phase/days		Chronic phase/days	
		1	4	7	14	30	≥ 60
Se-replete mice	0	5	5				5
	1			2	1	5	
	2				2		
	3			3			
	4						
	5				2		
Se-deficient mice	0	5	5				7
	1			2	1	4	1
	2			2	1	1	
	3				1		1
	4				1		
	5			1	1		

For each testing day up until 60 days p.i., 5 mice were tested from both Se-replete and Se-deficient groups. Se-deficient mice were tested on 60 days p.i. and an additional 4 mice were tested on 90 days p.i. (≥ 60).

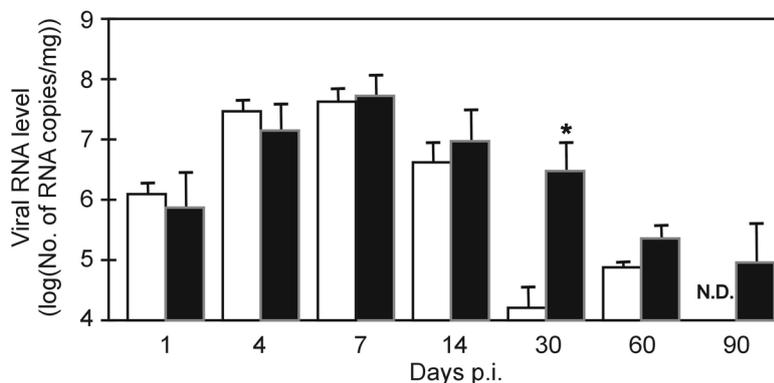


Fig. 4

Effect of Se diet on the viral RNA level in heart of the CVB3-infected mice quantified by real-time RT-PCR

Se-replete (empty columns) and Se-deficient (full columns) mice. N.D. = not done

to the chronic myocarditis that featured a sustained level of virus replication.

Discussion

Here, we provided evidence that i) Se deficiency increased the propensity toward chronic myocarditis following CVB3 infection, ii) CVB3 replication in the heart progressed to the chronic phase under Se-deficient conditions, and iii) there was a dramatic loss of the antioxidant GPx activity in chronic phase of the CVB3-infected Se-deficient mice.

Immune inflammatory infiltration is a recognized feature of CVB3-related myocarditis and plays a crucial role in the heart disease process (Woodruff, 1980; Leslie *et al.*, 1989). During the subacute phase from 7 to 14 days p.i., severe inflammatory infiltrates were observed in the heart tissue. A lesser degree of myocarditis alteration was still evident on day 30 p.i. regardless of the Se conditions. Thereafter, 22% of Se-deficient mice continued to experience chronic myocarditis changes up to 90 days p.i. During this later infection period, there were no pathologic characteristics in the mice fed a Se-replete diet.

Virus replication in tissues was quantified by determination of the viral genomic RNA concentration using a real-time RT-PCR. We have previously reported that the amount of viral RNA determined by this technique directly correlated with the infectious virus titer measured by plaque assay (Moon *et al.*, 2005). Because of the potential of PCR to detect genomic fragments, this technique offered a sensitivity advantage and could also be used for the analysis of older formalin-fixed samples to find an evidence of infectious agent (Mygind *et al.*, 2001). Altogether, the kinetics of virus replication determined by real-time RT-PCR was in accord with the myocarditis pathology characteristics. Virus repli-

cation was approximately the same in both groups during the acute and subacute phases that subsequently led to the severe inflammatory infiltration. Moreover, the hearts of Se-deficient mice maintained a significantly higher level of the virus in the chronic phase of infection during which myocarditis often persisted. This observation thus suggested that persistence of replication was closely associated with the chronic myocarditis that was preferentially observed under Se deficiency following CVB3 infection.

There is a large body of evidence implying that micronutrients such as vitamins and minerals play an important role in the immune response (Chandra, 1990; Good and Lorenz, 1992). In addition, randomized controlled trials have provided a strong evidence that multi-micronutrient supplementation effectively reduces the risk of various types of infection (Stephen and Avenell, 2006). In the context of current study, Se deficiency has been shown to attenuate dramatically the expression of several cytokines after virus infection, particularly interferon-gamma (Beck and Matthews, 2000). This could have implications for the viral potency to that extent that an ineffective production of interferon-gamma might directly allow the active replication of CVB3 during chronic and acute phase of the infection. In addition to the immune defects, other data demonstrated that Se deficiency could lead to the acquisition of virulence through a genotypic alteration of avirulent viruses (Beck *et al.*, 1994, 1995). It has been proposed that the enhanced virus replication under Se deficiency would inevitably increase the possibility of the viral mutations to occur. Thus, further accumulation of genetic changes can arise even in the CVB3 inoculum and potentially influence the virus pathology. However, this possibility was not addressed in the current study.

GPx activity was measured to evaluate the effect of a Se-deficient diet on the oxidative status of mice. As expected, there was no change in the GPx activity in mice fed on a diet

with adequate level of Se. In contrast, there was a significant loss of the GPx activity in mice fed on a Se-deficient diet. In chronically infected Se-deficient mice, virtually no enzyme activity was detected. It was demonstrated that GPx plays an important role in the scavenging of hydrogen peroxide and alkyl peroxides (Rotruck *et al.*, 1973). A state of oxidative stress imposed by Se deficiency could thus lead to an increase of free radicals that can damage cell membranes, cellular proteins, and nucleotide bases (Rivett, 1985; Davies *et al.*, 1987; Stadtman, 1992). An increase of free radicals might also negatively affect components of the immune system. Indeed, a study using GPx-knockout mice clearly showed that GPx protected mice from the CVB3-induced myocarditis (Beck *et al.*). Interestingly, GPx activity on day 1 p.i. was markedly increased in mice fed on the Se-replete diet implying an essential role of GPx in the host defense mechanism. Furthermore, our results indicated that the Se deficiency in diet contributed to the accelerated virus replication even during the chronic phase of CVB3 infection.

In conclusion, Se deficiency reduced GPx activity during the chronic phase of infection and allowed maintenance of a higher viral RNA level in the hearts of CVB3-infected mice. Consequently, higher level of the virus in CVB3-infected Se-deficient mice could promote the progression of myocarditis that was more advanced than the myocarditis observed in the CVB3-infected Se-replete mice.

Acknowledgements. This work was supported by the grants No. 2010-0743 from the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology and No. 2009-287 from the Asan Institute for Life Sciences to H. Lee.

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