The Ras antagonist farnesylthiosalicylic acid ameliorates experimental myocarditis in the rat

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Abstract

Background: Myocarditis is an inflammatory disorder of the heart in which T lymphocytes have a central role. No effective treatment is currently at hand for management of the myocarditis. Lymphocyte function requires the active signal transducer Ras. We thus hypothesized that S-farnesylthiosalicylic acid (FTS), a synthetic small molecule that detaches Ras from the inner cell membrane and induces its rapid degradation, will attenuate experimental autoimmune myocarditis (EAM). Methods and results: Two groups of Lewis rats were induced to develop EAM by immunization with porcine cardiac myosin. Group A received 5 mg/kg of FTS, and group B received phosphate-buffered saline (PBS) according to two protocols: FTS or PBS was given 2 days before myosin immunization in protocol 1 and FTS or PBS was given 14 days after myosin immunization in protocol 2. FTS significantly suppressed myocarditis, and this effect was accompanied by a reduction in myosin-specific cellular and humoral immune responses. In the longer regimen, FTS treatment for 6 weeks was associated with preservation of myocardial function made evident by echocardiography. In vitro, FTS significantly attenuated the proliferation of lymphocytes from untreated myocarditic rats to myosin. Conclusions: FTS is effective in suppressing the progression of EAM and its consequent functional myocardial dysfunction. The effect may be mediated by suppression of the cellular and humoral responses to myosin.

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1. Introduction

Myocarditis is an inflammatory heart disease and is potentially lethal in humans. The disease is characterized by immune cell infiltration in the myocardium, with potential subsequent development of fibrosis and myocardial dysfunction [1]. Myocarditis is caused by a variety of infections and systemic diseases [2] and can become autoimmune due to cross-reactive epitopes of cardiac myosin (mostly) and infectious agents. Although the precise mechanisms for the development of myocarditis are still unresolved, its pathogenesis has been suggested to involve natural killer cells, viral-specific cytotoxic T cells, and antimyosin antibodies [3–5]. Experimental autoimmune myocarditis (EAM) is a potentially useful animal model of myocarditis induced by immunization of rats or mice with cardiac myosin [6,7]. EAM in the rat follows a severe course that resembles the fulminant form of human myocarditis [7–9]. The onset of EAM is about 10 days after immunization, and the disorder is transferable by T cells [10,11]. If left untreated, EAM progresses toward dilated cardiomyopathy [12].

Ras protein is a signal transducer that has an important role in T-lymphocyte proliferation and activation [13]. Ras-dependent signaling requires both GTP binding and inner cell membrane anchorage. Specific anchorage of Ras is promoted by its carboxy-terminal S-farnesyl cysteine. S-Farnesylthiosalicylic acid (FTS; salirasib), an analog of
farnesylated Ras, destabilizes the attachment of Ras to the
cell membrane and is a potent inhibitor of Ras-mediated
signal transduction pathway [14]. Importantly, FTS exhibits
significant selectivity toward Ras in its active (GTP-bound)
form. Thus, FTS has the ability to disrupt Ras interactions
with the cell membrane in live cells without significant
cytotoxicity [15,16]. Animal studies [15] and initial human
trials (http://www.concordiapharma.com/index.htm) showed
that FTS is a relatively safe compound with no adverse side
effect. This property makes FTS a good candidate for clinical
use in immune-mediated disorders [14].

Little progress has been made in treating myocarditis by
immunosuppression because of insufficient understanding of
key factors that regulate the pathogenic immune responses in
autoimmune myocarditis.

Here, we demonstrate that FTS is effective in suppressing
the progression of EAM and suggest that the mechanism
mediating the protective effect of FTS may be related to its
influence on cellular immune responses.

2. Materials and methods

2.1. Animals

Male Lewis rats were purchased from Harlan and
maintained in a local animal house under conventional
conditions.

2.2 Induction of EAM

Porcine cardiac myosin (Sigma) was dissolved in
phosphate-buffered saline (PBS) to a concentration of
0.4 μg/μl. Each rat was immunized on day 0 with emulsion
containing 1 mg of cardiac myosin with an equal volume of
complete Freund’s adjuvant containing 10.0 mg/ml of heat-
killed Mycobacterium tuberculosis by a single subcutaneous
injection in both footpads.

2.3. FTS preparation for injections

FTS was synthesized as previously described [17]. FTS
was stored in chloroform, which was evaporated under a
stream of nitrogen immediately before use. The powder
was dissolved in absolute ethanol and diluted to the
desired concentration in sterile PBS made basic with
NaOH. Carrier solution (1000 μl) containing 1.35 mg of
FTS (5 mg/kg) was injected intraperitoneally into each rat.
Control solution was prepared at the same time starting
with PBS and absolute ethanol.

2.4 FTS injections

Two groups of rats were induced to develop EAM by
immunization with cardiac myosin. Group A received
5 mg/kg of FTS, and group B received PBS according to
two protocols: FTS or PBS was given 2 days before myosin
immunization and continued for 14 days in protocol 1 and
FTS or PBS was given 14 days after myosin immunization
and continued for 7 days in protocol 2. In both protocols,
FTS and PBS were given every other day. The rats were
sacrificed on day 21. Hearts, lymph nodes, and spleens were
taken for histological and immunological analyses.

In a third experiment, the effects of FTS in the prevention
of myocardial dysfunction induced following EAM were
studied. Thus, FTS or PBS was given 2 days before myosin
immunization and then every other day for 6 weeks (when
compromised heart function is expected); at that time point,
M-mode echocardiography was performed as previously
described [18].

2.5 Lymphocyte proliferation assays

Lymphocytes were obtained from lymph nodes of FTS-
and PBS-treated rats. The cells were washed, and 1×10^5 cells/
well were seeded in 96-microwell plates in RPMI medium in
triplicates. Cardiac myosin at 2.5 μg/ml and that at 5 μg/ml
were added to the medium. Concavalin A (4 μg/ml) was used
as a positive control for lymphocyte proliferation.

Lymphocyte proliferation was evaluated using XTT
[2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyla-
mino)carbonyl]-2H-tetrazolium hydroxide] reagent, accord-
ing to manufacturer’s instructions, after 24, 48, and 72 h
of incubation.

2.6. Determination of Ras, Erk, P-Erk, Akt, and P-Akt

Lymphocytes were obtained from lymph nodes of FTS-
and PBS-treated rats. The lymphocytes were homogenized in cold
homogenization buffer containing protease inhibitors. Protein
concentration was determined by the Bradford assay, and
samples containing 100 μg of protein were used for
determination of total Ras, total Erk, P-Erk, total Akt, and
P-Akt by Western immunoblotting using pan-anti-Ras antibody
(Ab03;Santa Cruz, CA), anti T-Erk antibody (Santa Cruz), anti
P-Erk antibody (Sigma), anti T-Akt antibody (Cell Signaling),
and anti P-Akt antibody (Cell Signaling), respectively.

Enhanced chemiluminescence and densitometric analyses
were performed as detailed previously [19].

2.7. Determination of Ras–GTP

The lymphocytes were prepared as mentioned above.
Samples containing 1.5 mg of protein were used for
determination of levels of active GTP-bound Ras by the
glutathione S-transferase–Ras binding domain pull-down
assay followed by Western immunoblotting with pan-anti-
Ras antibody as detailed previously [15].

2.8 Histopathology

Hearts were removed and fixed in 10% formalin and then
embedded in paraffin. Several transverse sections were cut
from the paraffin-embedded samples and stained with hematoxylin–eosin (H&E). Every fifth section was examined for the presence of myocarditis by light microscopy and evaluated according to previously published criteria describing the severity of myocarditis as follows: severe (2), >50% of the heart involved; moderate (1), 10%–50% of the heart involved; and minimal (0), <10% of the heart involved or normal.

Sections from each heart were also stained with Masson’s trichrome for an assessment of scar area.

2.9. FTS preparation for ex vivo assay

FTS was synthesized as previously described [17]. FTS was stored in chloroform, which was evaporated under a stream of nitrogen immediately before use. The powder was dissolved in DMSO. This solution was diluted with RPMI/10% fetal calf serum to yield 10 mM drug stock solution containing 10% DMSO. A portion of this solution was applied to the cells at a dilution of 1:100.

2.10 Ex vivo proliferation assay with FTS

Five rats with EAM were sacrificed on day 14. Lymphocytes were taken from lymph nodes, prepared as described, and 1×10^5 cells/well were seeded in 96-microwell plates.

Lymphocytes were incubated in the presence of 2.5 μg/ml of myosin or concavalin A in the presence of 0, 3.125, 6.25, 12.5, and 25 μM FTS. Lymphocyte proliferation was evaluated using XTT reagent after 96 h of incubation.

2.11. Immunoglobulin G antimyosin antibodies

Sera obtained from all rats at sacrifice were studied for antimyosin immunoglobulin G (IgG) antibody levels by enzyme-linked immunosorbent assay as previously described [20].

2.12. Statistical analysis

Results are expressed as mean±SE. Differences between groups were compared using Student’s t test. Differences were considered significant at P≤.05.

3. Results

3.1. The effect of FTS on lymphocyte proliferation ex vivo

We tested the effect of FTS on the proliferative capacity of lymphocytes from EAM rats against myosin ex vivo (Fig. 1). FTS (50 μM) gave maximal inhibition of proliferation after 96 h of incubation with both myosin and concavalin A (Fig. 1A and C). FTS dose dependently reduced lymphocyte proliferation to myosin (Fig. 1B); a strong and significant 69±11% inhibition was evident at 50 μM FTS (P<.05). Also, FTS dose dependently reduced
concavalin A-induced proliferation (Fig. 1D). The proliferation rate was reduced by 52% in the presence of 50 μM FTS (Fig. 1B). Trypan blue exclusion test demonstrated that cell viability was not compromised using the given FTS concentrations.

3.2. The effect of in vivo treatment with FTS on lymphocyte proliferation

In order to further investigate the effect of systemic FTS treatment on lymphocyte proliferation, we conducted in vivo experiments. FTS given 2 days prior to myosin immunization at a dose of 5 mg/kg and then for a period of 14 days (protocol 1, pretreatment before immunization) resulted in a significant 35% decrease in lymphocyte proliferation in comparison with PBS ($P < .05$) (Fig. 2). Moreover, the proliferation in response to the nonspecific mitogen concavalin A was not diminished in FTS-treated rats compared with the control group (not shown).

3.3. The effect of FTS on IgG antimyosin antibody levels

IgG antimyosin levels were then examined in order to evaluate the influence of FTS on the humoral response to myosin (Fig. 3). FTS given according to protocol 1 led to a significantly reduced level of IgG antimyosin antibody at serial dilutions. In addition, FTS given 1 week after myosin immunization for a period of 7 days (protocol 2) also significantly reduced IgG antimyosin antibody levels in the FTS-treated rats at serial dilutions.

3.4. FTS attenuates myocarditis and subsequent myocardial dysfunction

Heart sections were stained with H&E and the myocarditis score was evaluated using a scale of 0–2 arbitrary units (the Dallas criteria) to determine the severity of myocarditis. Rats that received FTS according to protocol 1 showed a significant reduction in the severity of myocarditis (0.28±0.15) compared with the control group (1.61±0.23) ($P < .05$; Fig. 4A). Rats that received FTS according to protocol 2 also showed a significant reduction in the severity of myocarditis (0.33±0.24) compared with the control group ($P < .05$; Fig. 4A).

In order to evaluate scar size, we used Masson’s trichrome staining. This staining allowed assessment of collagen deposition in the three study groups. Extensive deposition of collagen was observed in the control rats. FTS-treated rats from both protocols exhibited reduced deposition of collagen. Typical representatives of the stained sections and quantitative analysis of the results are shown in Fig. 4B and C.

Prolonged treatment with FTS significantly attenuated myocardial damage induced by EAM. This was made evident by improved systolic parameters such as fractional shortening and left ventricular diastolic diameter (Table 1).
3.5. The effect of FTS on Ras signaling pathways in lymphocytes of FTS-treated rats

In order to examine the effect of FTS on Ras and its prominent downstream effectors, we used Western immunoblotting. Quantitative analysis of the immunoblots disclosed that FTS reduced levels of total Ras, active Ras (Ras–GTP), p-Erk, and p-Akt in lymphocytes of rats from the protocol 1 group. Levels of Ras–GTP were reduced in lymphocytes of FTS-treated rats compared with the control group ($P<.05$). Levels of total Ras also decreased in lymphocytes of FTS-treated rats compared with the control group. Levels of p-Erk decreased in lymphocytes of FTS-treated rats compared with the control group ($P<.05$) although levels of total Erk did not differ between the two groups. FTS treatment was associated with a decrease in the levels of p-Akt as compared with the control group ($P<.05$), while levels of total Akt did not differ between the two groups (Fig. 5).

4. Discussion

Myocarditis is a severe, potentially lethal disease that is mostly induced by infectious agents. Current treatments
comprise various immunosuppressives that may be associated with further cardiac damage [21].

In the current study, we explored the possible therapeutic potential of the Ras inhibitor FTS in EAM. The results show that FTS injections, given as treatment or prevention therapy, were capable of ameliorating the progression of EAM. In addition, FTS also reduced lymphocyte reactivity to myosin ex vivo.

EAM is traditionally considered a T cell-mediated disorder [10,11], and data support this mechanism as involved in human myocarditis. As lymphocyte activation requires participation of several signal transduction pathways, with the Ras pathway being of principal importance [22], we reasoned that interfering with Ras functions would inhibit lymphocyte activation. Subsequently, proliferation of lymphocytes against potential cardiac epitopes may be attenuated following FTS administration.

Indeed, FTS treatment ex vivo showed reduction of lymphocyte reactivity against myosin (∼79% reduction at 50 μM FTS). In addition, lymphocytes treated with concavalin A also showed a dose-dependent reduction in proliferation that was smaller than that observed with myosin.

Table 1
Echocardiographic parameters in rats treated with FTS for 6 weeks

<table>
<thead>
<tr>
<th></th>
<th>Fractional shortening (%)</th>
<th>Left ventricular end-diastolic diameter (mm)</th>
<th>Left ventricular end-systolic diameter (mm)</th>
<th>End-diastolic interventricular septal thickness (mm)</th>
<th>End-diastolic posterior wall thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>76</td>
<td>27.7±3.3</td>
<td>6.4±0.4</td>
<td>3.5±0.8</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>FTS</td>
<td>87</td>
<td>36.2±3.1 **</td>
<td>4.7±0.7</td>
<td>3.6±0.89</td>
<td>1.29±0.2 *</td>
</tr>
<tr>
<td>Healthy</td>
<td>88</td>
<td>43.1±9.9 *</td>
<td>4.89±0.53 *</td>
<td>2.8±0.8</td>
<td>1.17±0.31 *</td>
</tr>
</tbody>
</table>

* P <.01; ** P <.05.

Fig. 5. Western immunoblotting analysis. (A) In order to examine the effect of FTS on Ras signaling, we performed Western immunoblotting on lymphocytes from control and FTS-treated rats (according to protocol 1). (B) Results of densitometric analysis of the immunoblots in both groups. Columns, mean±SE. *P<.05 compared with the control group.
The effects of FTS were studied on Ras and its downstream signaling effectors in lymphocytes from treated rats. The results presented here show that FTS down-regulated total Ras and Ras–GTP in lymphocytes from treated rats compared with the control animals. These results indicate that FTS did not significantly alter the ratio of Ras–GTP to total Ras. We know from earlier experiments that FTS exerts a direct effect on membrane-bound active Ras protein, dislodging it from the membrane, and has no effect on Ras farnesylation [19]. The present results are thus consistent with the known mechanism of action of FTS, in which a reduction in the content of active Ras results in a shift in the Ras–GTP/Ras–GDP steady state, leading to a depletion of total Ras. FTS not only decreased Ras protein levels but also had a functional effect on Ras signaling activity. FTS caused a significant decrease in the downstream effectors phospho-Akt and phospho-Erk, while total Erk and total Akt were not affected by FTS. This reduction is relevant since Erk and Akt signaling pathways are known to promote cell cycle progression and survival, enabling lymphocytes to thrive and become antigen responsive.

Our results show that FTS injections according to protocol 1 (prevention regimen) reduced the proliferative ability of myosin-reactive lymphocytes by ~35% compared with the control animals. Myosin-reactive lymphocytes are thought to migrate and infiltrate the heart, promoting cardiomyocyte damage and changes in the extracellular matrix. Thus, a reduction of lymphocyte reactivity against myosin may provide an explanation for the reduced myocarditis score and scar tissue in these animals. Previous experiments showed that FTS attenuated the course of experimental autoimmune encephalomyelitis [23], experimental autoimmune neuritis [16], and experimental lupus in MRL/lpr mouse [24]. Additionally, it has been shown that FTS ameliorates experimental restenosis in rats [25] and atherosclerosis in apolipoprotein E knockout mice [26]. Collectively, these studies support the notion that a suppressive effect on lymphocyte proliferation using FTS is potentially beneficial for the treatment of myocarditis. The role of humoral immune response in EAM is still controversial. While many studies show that cellular immunity is the principal player [10,11], there is also evidence of cardiac-specific autoantibodies influencing myocarditis progression [27]. Our results demonstrate, both in prevention and treatment protocols, a reduction in IgG antitymyosin antibody levels in comparison with the control group. Similar to our results, Katzav et al. [28] also showed that FTS treatment significantly lowered antibody levels in APS mice.

Although there is evidence that FTS influences Ras in lymphocytes [16,24], the beneficial effects of FTS are likely to occur by affecting the number of immune cells and their population of the target organs. In addition, isotype switch from IgM to IgG is T cell dependent; thus, attenuation of T-cell responses to myosin is associated with the decrease in antitymyosin antibody levels. These results are consistent with our observation that lymphocyte proliferation to myosin was indeed diminished by FTS treatment in vivo and ex vivo.

An additional finding of this study that is of particular relevance to the potential clinical application of FTS is its beneficial effects in preserving myocardial function. In the EAM model, myocardial performance gradually deteriorates due to replacement of the inflammatory infiltrate by a nonfunctional scar. It is likely that a robust inflammatory infiltrate is associated with a subsequent increased extent of myocardial damage. It is thus understandable that the reduction in lymphocyte infiltration by FTS made evident by a reduced myocarditis score was followed by functional improvement as observed in transthoracic echocardiography.

In conclusion, the present study shows that FTS was found to be effective in ameliorating EAM. The beneficial effect of FTS was associated with a reduction in the cellular and humoral immune responses to myosin. This supports the strategy of selective inhibition of intracellular signals as a potential therapy for myocarditis.

References


