Genistein down-modulates pro-inflammatory cytokines and reverses clinical signs of experimental autoimmune encephalomyelitis

Marcio L. De Paula, David H. Rodrigues, Henrique C. Teixeira, Michele M. Barsante, Maria A. Souza, Ana P. Ferreira

A Department of Parasitology, Microbiology and Immunology, Biological Sciences Institute, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil
B Department of Biochemistry and Immunology, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Received 29 February 2008; received in revised form 10 April 2008; accepted 5 May 2008

Abstract

Multiple sclerosis (MS) is the most common non-traumatic, disabling neurological human inflammatory demyelinating disease of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) models MS and is characterized as a CD4+ T-helper type 1 (Th1) cell-mediated autoimmune disease. It is characterized by an influx of activated leukocytes into the CNS. Genistein, occurring abundantly in soy products, has apoptotic, antioxidant, and anti-inflammatory properties. In the present report, we investigated the use of genistein for the treatment of the murine model of MS. After induction of EAE with myelin oligodendrocyte glycoprotein 35–55 peptide (MOG35–55), we observed that genistein treatment ameliorated significantly the clinical symptoms, modulating pro- and anti-inflammatory cytokines. Moreover, we analyzed the leukocyte rolling and adherence in the CNS by performing intravitral microscopy. Genistein treatment resulted in decreased rolling and adhering of leukocytes as compared to the untreated group. Our data suggest that genistein might be a potential therapy for MS.

© 2008 Elsevier B.V. All rights reserved.

KEYWORDS
Multiple sclerosis; Experimental autoimmune encephalomyelitis; Genistein; Cytokines; Intravital microscopy

1. Introduction

MS is a chronic inflammatory disorder of the CNS with unknown etiology affecting approximately 2.5 million people worldwide [1,2]. A complex predisposing genetic trait and an inciting environmental insult such as infections agents appear to be important in triggering the disease, comprising as
hallmarks inflammation, demyelination and axonal damage [1,3]. Cytokines such as TNF-α, IFN-γ, IL-17 and IL-12, and to some extent chemokines play a pivotal role in the establishment and maintenance of autoimmune disorders, acting in highly complex networks, and often exert overlapping and in part redundant functions by different cell types. Also, pro-inflammatory cytokines are thought to play a role in the pathogenesis of MS [4,5]. Due to its similarity to MS, EAE has been used as an animal model for proof of concept studies of MS therapy.

Both clinical and experimental evidence suggests that sex hormones in males and females are significant factors responsible for the sexual dimorphism in the immune response. The best-studied regulatory hormone, estrogen, is protective against the induction of EAE, demonstrating several immunosuppressive mechanisms. Phytoestrogens are a group of biologically active plant substances with a chemical structure similar to estrogen, exerting estrogenic and anti-estrogenic effects [6]. Previous data suggest that the consumption of phytoestrogens leads to protective effects against menopausal symptoms and a variety of disorders, including diabetes and cancer [7,8]. Isoflavones make up the most common form of phytoestrogens and are found in a variety of plants, especially in soy [9]. Genistein is the major bioactive isoflavone, demonstrating a variety of properties such as induction of apoptosis in cancer cells and antioxidant effects [10,11]. However, little has been elucidated with regard to its potential in autoimmune diseases caused by T cell activation.

IFN-β has been approved for treatment of relapsing–remitting MS and is currently the agent that is most broadly used as an immunomodulatory and suppressive treatment. It reduces exacerbation by only 30% and has a modest impact on disease progression. Indeed, it is a clear step forward in MS therapy, but the frequency of subcutaneous injections of IFN-β, the flu-like symptoms that occur at the beginning of therapy, the modest activity required of patients, and the treatment failures are all reasons to search for better agents [5]. In this report, we sought to determine the clinical and biological effects of isoflavone genistein on the myelin MOG-induced EAE model.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice 8–12 weeks old were obtained from the Animal Care Facilities of the Federal University of Juiz de Fora (UFJF) and housed in microisolator cages in the animal facility at the Laboratory of Immunology. All procedures were in accordance with the principles of the Brazilian Code for the Use of Laboratory Animals. This project was approved by the Ethics Committee on the use of laboratory animals from UFJF.

2.2. EAE induction

Groups of 3–6 animals were immunized or not subcutaneously (s.c.) at both sides of the tail base with 100 μg of MOG 35–55 peptide (Sigma Chemical Co., Saint Louis, USA) emulsified vol/vol in complete Freund’s adjuvant (CFA) (Sigma) supplemented with 400 μg of attenuated Mycobacterium tuberculosis H37 RA (Difco, Detroit, USA). Pertussis toxin, 300 ng/animal (Sigma), was injected intraperitoneally (i.p.) on the day of immunization and again 48 h later. Non-immunized animals were used as control group. Animals were monitored daily and neurological impairment was quantified on an arbitrary clinical scale.

2.3. Clinical assessment

Mice were weighed and observed daily for clinical signs of EAE up to 21 days post-immunization (dpi). The clinical status was assessed scoring certain parts of the mice body individually according to Table 1. In case of death, mice were scored 15. The final clinical score was obtained adding all individual scores assessed.

2.4. Treatment with genistein

Mice were immunized and then divided into two groups. One received no treatment while the other was given 200 mg/kg body weight of genistein (Indofine Chemical Co., Hillsborough, USA) in dimethyl sulfoxide (DMSO) (Sigma) 4% s.c. on daily basis [12]. The treatment was introduced 14 dpi for 7 days. In addition to the non-immunized control group that also received no genistein treatment, we used another non-immunized group in which DMSO 4% was applied only.

2.5. Cytokine production in the CNS

Brain tissue extracts were acquired from control and experimental mice that were sacrificed by ketamine and xylazine overdose i.p. at 21 dpi. Brains were removed after intravital microscopy, and hemispheres were stored on ice. Thereafter, the hemispheres were homogenized in extraction solution (100 mg of tissue per 1 ml), containing: 0.4 M NaCl, 0.05% tween 20 (Merck Co., Inc., Whitehouse Station, USA), 0.5% bovine serum albumin (BSA), 0.1 M phenylmethyl-sulphonyl fluoride (PMSF), 0.1 M benzethonium chloride, 10 mM ethylenediaminetetraacetic acid (EDTA) and 20 kIU/ml aprotinin (Sigma), using Ultra-Turrax (IKA Works, Wilmington, USA). Brain homogenate was spun at 10,000× g for 10 min at 4 °C and supernatants were collected and stored −70 °C. The concentration of IFN-γ, TNF-α, IL-12p40 and IL-10 in the supernatants of brain extraction, at 1:10 dilution in 1% BSA in phosphate buffered saline (PBS), was assayed in an ELISA set-up using commercially available antibodies and the concentrations according to the procedures supplied by the manufacturer (BD Biosciences Pharmingen, San Diego, USA).

2.6. Splenocyte culture

Suspensions of splenocytes were prepared on day 21. Cells were cultured at a density of 2×10^6/well in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM

<table>
<thead>
<tr>
<th>Part of the body</th>
<th>Clinical signs</th>
<th>Score a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail</td>
<td>No clinical signs</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Loss of muscle tone in tail</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Paralysis</td>
<td>2</td>
</tr>
<tr>
<td>Hind-limb</td>
<td>No clinical signs</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Weakness of one animal paw</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Weakness of both animal paws</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Paralysis of one animal paw</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Paralysis of both animal paws</td>
<td>4</td>
</tr>
<tr>
<td>Front-limb</td>
<td>No clinical signs</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Weakness of any animal paw</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Paralysis of any animal paw</td>
<td>2</td>
</tr>
<tr>
<td>Bladder</td>
<td>Continence</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Incontinence</td>
<td>1</td>
</tr>
</tbody>
</table>

a Numerical score arbitrarily established.
L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI 5%, Sigma), in a humidified incubator, at 37 °C and at 5% CO₂ atmosphere. Cultures were stimulated with MOG35–55 peptide at the final concentration of 10 μg/ml, concanavalin A (Sigma) at 10 μg/ml, or genistein at 10 μg/ml. Supernatants were collected after 48 h and a quantitative ELISA was performed for IFN-γ, IL-10 and TNF-α according to the manufacturer’s recommendation (BD Biosciences Pharmingen).

2.7. Intravital microscopy

Intravital microscopy of the mouse cerebral microvasculature was performed as previously described [13]. Briefly, the mice were anesthetized i.p. with a mixture containing 150 mg/kg ketamine and 10 mg/kg xylazine and the tail vein was cannulated for administration of fluorescent dyes. A craniotomy was performed using a high-speed drill (Dremel, New York, USA) and the dura mater was removed to expose the underlying pial vasculature. Throughout the experiment, the mouse was maintained at 37 °C with a heating pad (Fine Science Tools Inc., North Vancouver, Canada) and the exposed brain was continuously superfused with artificial CSF buffer, an ionic composition containing, in mmol/L: NaCl 132, KCl 2.95, CaCl2 1.71, MgCl2 0.64, NaHCO3 24.6, dextrose 3.71 and urea 6.7; pH 7.4, at 37 °C. To observe leukocyte–endothelium interactions, leukocytes were fluorescently labeled by intravenous administration of rhodamine 6G (0.5 mg/kg body weight) and observed using a microscope BX201 (Olympus, New York, USA), X20 objective lens (Olympus), corresponding to 100 Am of area, outfitted with a fluorescent light source (epi-illumination at 510–560 nm, using a 590-nm emission filter). A silicon-intensified camera DEI-470 (Optronics Engineering,

Figure 1  Genistein ameliorates EAE clinical signs. Animals were monitored daily for clinical signs of EAE after immunization with 100 μg MOG35–55 peptide. Mice were treated (n=6) or not (n=6) with 200 mg/kg body weight of genistein during 7 days. (A) Weight measurement of EAE mice treated or not with genistein, and from the control (n=6). (B) Clinical scores of EAE mice treated or not with genistein at 10 dpi. Each point represents the arithmetic mean ± SEM, and results are representative of two different experiments. Dashed lines: beginning of the treatment. *, p<0.05; **, p<0.01; ***, p<0.001.
Goleta, USA) mounted on the microscope projected the image onto a monitor (Olympus). Rolling leukocytes were defined as white cells moving at a velocity less than that of erythrocyte cells. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 s or longer.

2.8. Statistical analysis

Results presented here represent at least two independent experiments and are presented as the mean ± SEM. For clinical score and weight measurement analysis, two-way ANOVA was performed while the others were assessed by one-way ANOVA. All analyses were followed by the Bonferroni Multiple Comparison test (GraphPad Prism 5.00), and the differences were considered significant at \( p < 0.05 \).

3. Results

3.1. Treatment with genistein ameliorates the EAE clinical course

The initial clinical expression of the axonal damage in an EAE model is represented by well-defined signs such as weight loss, tail paralysis and hind-limb weakness [14]. In this work, we investigated whether genistein would be effective in the treatment of EAE induced in C57Bl/6 mice. These animals were immunized with MOG\(_{35-55}\) peptide in adjuvant, and a group was treated daily with genistein 3 days after the beginning of the clinical signs (14 dpi), when the disease was already established. EAE severity was recorded daily using a weight measurement and a clinical score scale. The immunized mice presented disease signs of EAE such as weakness or paralysis of their tail and limbs as well as loss of body weight, which became apparent around days 10–12 following immunization. Subcutaneous injections of genistein were demonstrated to be effective in treating MOG\(_{35-55}\)-induced mice. From day 12 on, the daily weight means of untreated animals were remarkably different when compared to control animals \( (p < 0.001 \) from day 13 to 21) (Fig. 1A). Thus, the treatment with this compound was revealed to be significantly more efficient at days 20 \( (p < 0.05) \) and 21 \( (p < 0.01) \), as genistein-treated and untreated groups were compared (Fig. 1A). On the other hand, while the weight mean for the untreated mice remained lower than for the genistein-treated mice at the last dpi, genistein-treated mice increased in weight and recovered initial weight at the endpoint (Fig. 1A). Clinical evidence of disease in the genistein-treated group (first noticed at day 10) peaked at day 15 after immunization while in the untreated group it peaked at days 17 and 18 according to the clinical score assessment (Fig. 1B). From day 17 on, the groups showed pronounced differences mainly at the last four time points which were statistically significant \( (p < 0.05) \) (Fig. 1B). Limp tail and hind-limb weakness were the major clinical features noted. Paralleling the clinical signs after treatment with genistein, there was not only a dramatic weight gain but also a reduction of the clinical score in the treated group at the same time (Fig. 1).

3.2. Genistein modulates EAE by up-regulating IL-10 and down-regulating inflammatory cytokines in the CNS

To determine the cytokine profile, we measured IFN-\( \gamma \), TNF-\( \alpha \) and IL-10 levels in the brain and on the splenocyte supernatants at day 21 post-immunization. IL-12 was also assessed in the

![Figure 2](https://example.com/figure2.png)

**Figure 2** Production of IFN-\( \gamma \), IL-12, IL-10 and TNF-\( \alpha \) cytokines in the brain from mice immunized with 100 \( \mu \)g MOG\(_{35-55}\) peptide and treated \( (n=6) \) or not \( (n=6) \) with 200 mg/kg body weight of genistein at 21 dpi, and from the control \( (n=6) \). Each bar represents the arithmetic mean ± SEM, and results are representative of two different experiments. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).
brain. Higher levels of IFN-γ and IL-12 were observed in the untreated group than in the control group ($p < 0.05$) (Fig. 2). Importantly, an impressive suppression not only of IFN-γ but also of IL-12 cytokine in the brain of the mice treated with genistein in comparison to untreated mice ($p < 0.01$) (Fig. 2). Likewise, even in relation to control mice, reduced IFN-γ levels were found in the treated mice ($p < 0.05$) (Fig. 2A). Conversely, elevated IL-10 levels were noted in the brain of the genistein-treated mice when compared to untreated ($p < 0.01$) and control groups ($p < 0.05$) (Fig. 2C). The TNF-α assessment in the brain revealed much lower levels in the genistein-treated mice ($p < 0.001$) compared to the untreated group (Fig. 2D). Surprisingly, no significant levels of TNF-α were observed in the brain of the untreated mice in relation to control mice (Fig. 2D).

Figure 3 Production of IL-10, IFN-γ and TNF-α cytokines by ConA-driven splenocytes from mice immunized with 100 μg MOG35-55 peptide and treated ($n=6$) or not ($n=6$) with 200 mg/kg body weight of genistein at 21 dpi, and from the control mice ($n=6$). Each bar represents the arithmetic mean±SEM, and results are representative of duplicate cultures from two experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.3. Leukocyte rolling and adherence are remarkably altered by genistein treatment

The leukocyte–endothelium interactions in the pial microcirculation of EAE mice treated or not with genistein were analyzed using intravital microscopy at day 21 post-immunization. Firstly, assessing the number of rolling leukocytes, we found significant increased events in the untreated group in comparison to the control group ($p < 0.05$) (Fig. 4A). In the genistein-treated mice, reduced rolling leukocytes were observed following EAE induction as compared to untreated mice ($p < 0.05$) (Fig. 4A). Secondly, we noted an increased number of adhering leukocytes in the untreated animals in relation to the control animals ($p < 0.001$) (Fig. 4B). Also, in the animals treated with genistein, there was a marked increase of these events as compared to untreated mice ($p < 0.001$) (Fig. 4B).

Figure 4 Visualization of leukocyte–endothelium interaction from mice immunized with 100 μg MOG35-55 peptide and treated ($n=4$) or not ($n=4$) with 200 mg/kg body weight of genistein at 21 dpi, and from the control ($n=3$). Intravital microscopy was used to assess the rolling (A) and firm adhesion (B) of leukocytes/ min on brain microvasculature. Each bar represents the arithmetic mean±SEM, and results are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 

Specific antigens to assess cytokine levels on supernatants. Not only IFN-γ but also IL-10 production diminished in cultures from genistein-treated mice in response to ConA which stimulates polyclonal T cell response, as shown in Fig. 3A ($p < 0.01$) and 3B ($p < 0.05$). Also, the TNF-α production was reduced in the genistein-treated group as compared to the untreated group, although with no statistical significance (Fig. 3C). In response to MOG35-55 peptide and genistein, no differences in regard to cytokine profile were detected in vitro (data not shown).
4. Discussion

MS is still considered a CD4+ Th1-mediated autoimmune disease, which has socioeconomic importance second only to trauma in young adults [5]. Although a number of immunomodulatory and immunosuppressive agents have been applied to MS treatment, better defined therapeutic strategies are required. Flavonoids and phytoestrogens have been tested in the EAE murine model, demonstrating that these compounds may have beneficial properties [15–17]. Due to genistein being the major bioactive isoflavone in soybeans, the biological properties of the phytoestrogens have been attributed to it. Substantial data demonstrate that genistein acts not only at the molecular level inhibiting the activity of enzymes utilizing ATP such as tyrosine-specific protein kinases but also at the cellular level inducing apoptosis and inhibiting cell proliferation, suppressing osteoclast and lymphocyte functions, and exerting antioxidant effects [11,18].

Herein, we investigated the effects of this compound on the EAE model by triggering the clinical disease in C57BL/6 mice through injection of MOG. We noted that genistein had significant beneficial effects on EAE decreasing severity not only by down-regulating inflammatory cytokines but by affecting the trafficking of leukocytes into CNS as well.

Animal models of autoimmune diseases are providing a valuable means of analyzing the functional roles of cytokines in the pathogenesis of autoimmunity. In our study, genistein treatment significantly lowered pro-inflammatory cytokine levels such as IFN-γ and IL-12 in the CNS. In addition, IFN-γ production was diminished on ConA-driven splenocytes. IFN-γ activates microglia to act as effector cells that damage CNS cells via phagocytosis and the release of citotoxic factors including glutamate, NO, superoxide, and pro-inflammatory cytokines [19]. Conversely, IFN-γ-knockout animals develop normal EAE compared to wild-type littermates [20]. Despite IFN-γ possibly playing a relevant role in remission of MS by up-regulating pro-apoptotic proteins [21], the prevailing perception is that IFN-γ plays important roles in the initiation and development of MS. IL-12, a main stimulator of IFN-γ, has long been known to drive Th1 polarization [22], and use of anti-IL-12 can ameliorate EAE [23]. Yet in the absence of IL-12Rβ2, mice developed more severe clinical signs [24]. At least in part, IL-12 has been shown to act on the disease initiation and in the CNS inflammatory events of MS [19].

Next, we evaluated the local TNF-α levels in brain supernatants. TNF-α production is associated with Th1 response and classically induces activation of a variety of cell types and expression of adhesion molecules, cytokines, and chemokines in the CNS, which leads to CNS invasion by T cells and monocytes. The expression of TNF-α in the CNS parallels the disease course in EAE [4]. In TNF-α knockout mice, invasion of the CNS by immune cells is delayed, but later develops extensively. In humans, elevated serum TNF-α concentrations and PBMC secreting TNF-α have been reported in MS patients [25]. According to kinetic studies in the EAE model, this cytokine peaks when clinical signs and T cell infiltration are remarkable, and declines thereafter. Also, TNF-α can be produced by different cell types at distinct times in the disease process [26]. It may explain similar TNF-α amounts found here in control and untreated groups at 21 dpi. Besides IFN-γ and IL-12, genistein dramatically reduced TNF-α levels after 7 days of treatment in comparison to the untreated group. Therefore, suppressed local TNF-α levels suggest a remarkable effect of genistein in MOG-induced EAE.

In order to rule out the possibility of genistein’s vehicle interfering with the results, we applied DMSO on non-immunized animals. Similar results to control group were noted when only DMSO was used (data not shown). In the present report, we also analyzed IL-10 levels in the CNS and on culture splenocyte supernatants. Reduced IL-10 levels on ConA-driven cultured splenocytes may be explained by different responses in distinctive cells [27]. IL-10 has as primary function to inhibit cytokine production by macrophages such as TNF-α [28]. Genistein may diminish TNF-α levels either directly, or indirectly by augmenting IL-10 expression. Accumulated evidence has demonstrated that IL-10 is an important cytokine in the recovery from EAE. In contrast, IL-10 was shown to play a dominant role in the regulation of EAE since IL-10−/− mice suffer a severe non-remitting disease [28]. Nevertheless, the consensus is that IL-10 is the most potent regulator of EAE [29], and a cytokine circuit involving IL-10 and IL-12 as counter-regulators has been proposed to control disease progression [30]. In this context, compounds such as genistein might become useful in inflammatory disorders including MS.

A pathological hallmark of MS is the infiltration of immune cells across the endothelium of the blood brain barrier and their subsequent entry into the CNS [5]. Leukocyte extravasation has been separated into discrete steps, which are associated with interacting pairs of selectins and their ligands, integrins and cell-adhesion molecules, and particularly chemokines and their receptors [31]. To migrate into sites of inflammation, leukocytes must first roll along the vessel before they firmly adhere and emigrate out of the microvasculature [32]. In the current work we performed intravital microscopy to evaluate the leukocyte rolling and adhesion in the brain microvasculature of EAE mice. Previous studies have demonstrated the presence of chemokines in brain lesions and CSF of MS patients [33,34]. We noted a marked alteration not only in leukocyte rolling but also in leukocyte adherence, suggesting that genistein acts indirectly on the interaction between cells and endothelium in the pial microvasculature by down-regulating inflammatory cytokines as discussed above. Even though we did not evaluate the determinant molecules involved in this process such as chemokines, integrins or cell-adhesion molecules, we cannot rule out that genistein might also act directly on them especially because phytoestrogens have significantly higher affinities for estrogen receptors-β, which are located in high concentration in the brain [35]. In addition, experimental evidence reported that genistein inhibits important enzymes responsible for up-regulating adhesion proteins and integrins [36].

Taken together, our results shed more light on potential alternative therapies that might be applied to MS. Limiting the activation and migration of immune cells into the CNS appears to be an interesting approach to developing a novel treatment for MS.

Acknowledgments

This work was supported by grants from CAPES, CNPq and FAPESP. Language assistance was provided by Daniel Stockdell.
References


