Phytoestrogen genistein as an anti-staphylococcal agent

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Abstract

The soybean-derived isoflavone genistein has been shown to exert beneficial effects on many disorders, including cancer and cardiovascular diseases. The effects of genistein on mammalian cells are mediated by its abilities to inhibit topoisomerase II and protein tyrosine kinase. In order to examine the potential antibacterial activities of genistein, we incubated the bacteria with various concentrations of this compound for different periods of time and assessed the viable counts. Exposure to genistein exhibited an inhibitory effect on all staphylococcal strains tested, including methicillin-resistant strains. Furthermore, the growth of Streptococcus pasteurianus, Bacillus cereus, and Helicobacter pylori was clearly inhibited by genistein, whereas Escherichia coli growth was not suppressed. Daidzein, which is structurally similar to genistein, but deficient in topoisomerase II inhibitory activity, also inhibited the growth of Staphylococcus aureus, albeit with lower potency than genistein. Our results indicate that genistein exerts potent antibacterial properties in vitro, which are possibly mediated by the stabilization of the covalent topoisomerase II-DNA cleavage complex.

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Keywords: Genistein; Topoisomerase II inhibitor; Staphylococcus aureus

1. Introduction

The isoflavonoid genistein is a phytoestrogen that is found at high levels in soy products. Extensive epidemiological and animal studies and in vitro experiments with genistein have indicated beneficial effects of this compound on a multitude of disorders, including cancer [1,2] cardiovascular diseases [3], osteoporosis, and postmenopausal symptoms [4]. In addition, genistein has been widely used as a protein tyrosine kinase (PTK) inhibitor that blocks LPS-induced release of IL-6 and TNF in vitro [5] and in vivo [6]. Several studies have demonstrated that the adhesion and internalization of Staphylococcus aureus [7–9] and Escherichia coli [10] are reduced by the PTK-inhibitory activities of genistein.

The therapy of bacterial infections often involves antibiotics. However, due to the increasing prevalence of antibiotic-resistant bacteria, the search for new antibacterial compounds has attained a high priority. Phytoalexins, which are the best-known isoflavonoids, are known to inhibit the growth of pathogenic microorganisms in plants [11]. The antibacterial activity of flavonoids against S. aureus, including antibiotic-resistant strains, and Staphylococcus epidermidis has been reported recently [12], and there is an increasing interest in the use of plant flavonoids for treating human diseases. The prokaryotic type II topoisomerases (DNA gyrase and topoisomerase IV) are targets for broad-spectrum antibiotics [13]. Genistein is a topoisomerase II inhibitor, and has been shown to stimulate topoisomerase IV-mediated DNA cleavage in E. coli [14]. Since the impact of genistein on bacterial growth and on infectious diseases is unknown, we investigated the antibacterial activities of this compound. To examine whether genistein could directly inhibit bacterial growth, different strains of bacteria were cultured in broth that contained various concentrations of genistein. Interestingly, the growth of all of the S. aureus strains tested, including the methicillin-resistant (MRSA) strains, was sensitive to genistein. In addition, the growth of the other bacterial species tested, except that of E. coli, was inhibited by genistein.

2. Materials and methods

2.1. In vitro incubation of bacteria with genistein and daidzein

We analyzed the effects of genistein on the following bacterial species: S. aureus LS-1, Newman, 67-0 (MRSA),...
2.2. DNA methylation patterns of genistein-treated S. aureus

*S. aureus* strain LS-1 was treated with genistein at concentrations of 0, 10, 100 and 200 µM for 2.5 h at 37 °C. Genomic DNA was isolated from the bacteria and digested with the *Hpa*II (methylation sensitive) and *Msp*I (methylation resistant) restriction enzymes. The samples were electrophoresed on a 1% agarose gel and visualized under UV light following ethidium bromide staining.

2.3. Treatment of staphylococcal infection with genistein

The 6- to 8-week-old NMRI female mice, obtained from B&K Universal AB (Stockholm, Sweden), were housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg under standard conditions of light and temperature, and were fed standard laboratory chow and water ad libitum. The experiments were performed with the approval of the Ethical Committee of Göteborg University.

The *S. aureus* strain LS-1 bacterial stock was thawed and washed in PBS. Viable counts were performed to check the number of bacteria. The mice were given i.v. injections (in the tail vein) of 0.8–1.5 × 10^7 bacteria in a volume of 0.2 ml. Genistein was dissolved in DMSO and frozen in aliquots. The genistein stock solution was diluted in propylene glycol. The injected volume was 100 µl, which contained 5% DMSO. Two separate experiments were performed. The mice were injected subcutaneously (every second day) with either 30 or 60 mg genistein per kg body-weight, starting 2 days prior to bacterial inoculation. The control animals received the vehicle (DMSO and propylene glycol). The concentration of genistein used in these experiments was chosen based on earlier studies [6,18,19].

2.4. Clinical evaluation of mortality and arthritis

All of the mice were monitored individually. Their limbs were inspected visually every second day of the experiment. Arthritis was defined as visible joint erythema and/or swelling of at least one joint. To evaluate the intensity of arthritis, we used a clinical scoring system of 0–3 points for each limb (1 point, mild swelling and/or erythema; 2 points, moderate swelling and erythema; 3 points, marked swelling and erythema). The arthritic index was derived by adding the scores for all four limbs of each animal and dividing by the number of animals.

2.5. Determination of bacterial growth

Ten days after bacterial inoculation, the kidneys were aseptically removed. Appropriate dilutions were made and 0.1-ml aliquots of the tissue suspensions and of whole blood were plated on agar plates that contained 5% horse blood. After incubation for 48 h at 37 °C, the bacterial colonies were counted and tested for catalase and coagulase activity.
3. Results

3.1. Genistein inhibits in vitro growth of S. aureus

Initial experiments were performed using genistein at concentrations of 1, 10, 50, 100 and 370 µM. These concentrations were chosen based on earlier studies [7,10,14,20]. Genistein concentrations of 1 and 10 µM did not inhibit bacterial growth, and 370 µM induces cell death and cell lysis in eukaryotic cells [21,22]. In the following experiments, we used genistein concentrations of 50 and 100 µM. Approximately 10^3 CFU/ml of S. aureus strain LS-1 were added to genistein- or vehicle-containing broth. The cultures were incubated for 2, 5, 10, 15 and 24 h. Maximal inhibition was obtained with an incubation time of 10 h (Fig. 1). After 15 h of incubation, there was no significant effect of genistein on bacterial viability, possibly due to the degradation of genistein in the culture, or outgrowth of resistant bacteria.

The growth of S. pasteurianus, B. cereus, and S. aureus strains LS-1, SKM 7 (srtB), SKM 12 (srtA-), SKM 14 (srtA-srtB-), and Newman was reduced 2- to 160-fold by the addition of 100 µM genistein. By the same token, the growth of the MRSA strains 67-0, 1061, and Pls was inhibited 11- to 26-fold by genistein (Fig. 2). When log-phase S. aureus LS-1 bacteria was added to genistein-containing broth, there was a fivefold decrease in bacterial numbers (Table 1).

3.2. Genistein inhibits growth of H. pylori but not E. coli

In addition to the analysis of Gram-positive species, two Gram-negative species, H. pylori and E. coli, were tested for growth inhibition by genistein. Interestingly, the growth of E. coli was not inhibited (genistein-exposed: 88 × 10^6 vs. control: 10^7 × 10^6 CFU/ml), but the growth of H. pylori strains 17874 (genistein-exposed: 19 × 10^4 vs. control: 176 × 10^4 CFU/ml), and Hel 340 (genistein-exposed: 35 x10^4 vs. control: 176 × 10^4 CFU/ml) was inhibited by the addition of 100 µM genistein.

3.3. Impact of daidzein, a structurally similar compound to genistein, but having deficient topoisomerase II inhibition properties, on staphylococcal growth

The chemical structure of daidzein is very similar to that of genistein, except that it lacks the -OH group at position 5. Although daidzein is found in the same types of plant and has similar activities as genistein, it is not an inhibitor of protein tyrosine kinases and, as such, is often used experimentally as the inactive form of genistein. In addition, genistein inhibits the function of the DNA topoisomerase II enzyme [23], whereas daidzein has been shown not to induce DNA strand breaks in cultured cells [24]. Our results indicate that equimolar amounts of daidzein display intermediate levels of S. aureus LS-1 growth inhibition, i.e. daidzein had a less pronounced effect on growth than genistein, when log-phase bacteria (Fig. 3A,B) or bacteria that were thawed from frozen stocks were used (Fig. 4A,B).

3.4. DNA methylation patterns of genistein-treated S. aureus

Since the genomic DNA of S. aureus contains unmethylated cytosines in the CCGG motif, it undergoes digestion by both HpaII and MspI. There were no obvious differences in the restriction enzyme digestion patterns of the genomic DNA between the genistein-treated bacteria and the untreated controls (data not shown), which suggest that the gross cytosine methylation pattern of the bacterial genome is not altered by treatment with genistein.

### Table 1

<table>
<thead>
<tr>
<th>Inoculum of S. aureus</th>
<th>Genistein</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>1 × 10^7 CFU/ml</td>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td>1 × 10^8 CFU/ml</td>
<td>8</td>
<td>40</td>
</tr>
</tbody>
</table>

Data are expressed as x10^7 CFU/ml.

*Inocula of bacteria grown to mid-log phase were added into 100 µM genistein- or vehicle-containing broth.
3.5. Impact of genistein administration on *S. aureus*-induced arthritis

Two experiments were performed, in which *S. aureus*-infected mice were treated with genistein. The *S. aureus* LS-1 inocula varied between $0.8 \times 10^7$ and $1.5 \times 10^7$ CFU/mouse. In the first experiment, mice were treated with 30 mg of genistein per kg body weight. No statistically significant differences were noticed between the groups with respect to the progress or outcome of clinically observed arthritis (data not shown). Mortality was somewhat lower in the genistein-treated mice than that in the controls (30% vs. 50%). In the second experiment, mice were administered 60 mg of genistein per kg body weight, following the same regimen as in the first experiment. In this instance, a tendency to less severe (Fig. 5A) and less frequent (Fig. 5B) arthritis was observed in the genistein-treated mice. The bacterial numbers in the kidneys and blood were similar in all of the infected mice in both experiments.

4. Discussion

In recent years, phytoestrogens have attracted increased attention because of their potential to afford protection against a variety of disorders, including cancer, hyper-
lipemias, osteoporosis, cardiovascular diseases, and various forms of chronic renal disease [25]. The most abundant food sources of isoflavones are soybeans and soybean products. The major bioactive isoflavones are genistein and daidzein [26]. In this study, we demonstrate that genistein inhibits the growth of S. aureus strains in vitro, which include MRSA strains. A similar outcome was observed with the other Gram-positive bacteria tested. In contrast, the inhibitory effects of genistein on Gram-negative bacterial growth were variable and species dependent. We found that genistein inhibited the growth of H. pylori, but not that of E. coli. Bae et al. [20] recently demonstrated the inhibitory effect of genistein on H. pylori growth. Another study by Susa and Marre [27] showed that genistein treatment of Legionella pneumophila did not influence the bacterial viability.

Two bacterial type II topoisomerases have been identified: topoisomerase IV (topo IV) and gyrase. Topo IV catalyzes the separation of two double-stranded covalently closed circular DNA molecules that are intertwined in a chain. Several inhibitors of DNA gyrase and/or topo IV have been developed for clinical use as antimicrobials. Synthetic antibiotics target both gyrase and topo IV by stabilizing the topo II-DNA reaction intermediate, known as the cleavable complex. By trapping this complex, they introduce lesions into the intracellular DNA. However, the relatively high-level cytotoxicity of these drugs, and the tendency of bacteria to develop resistance, limits the use of these antibiotics. Genistein inhibits the catalytic activity of the enzyme by stabilizing the covalent topoisomerase II-DNA cleavage complex [23]. We propose that genistein inhibits bacterial growth in vitro by the inhibition of topo IV, based on several lines of evidence. First, genistein inhibits the activity of topo IV rather than that of gyrase [14]. Second, topo IV in S. aureus is the primary target of topo II-targeted drugs, while in E. coli the primary target is gyrase [28]. Our results demonstrate genistein-mediated growth inhibition of S. aureus but not of E. coli. In addition, daidzein, which is described as a weak topo II inhibitor [29], but which lacks PTK inhibitory activity [30], exerted antibacterial activity, although it was less potent in this regard than genistein.

The biological significance of tyrosine phosphorylation has been extensively characterized in eukaryotes. In contrast, tyrosine phosphorylation in prokaryotes is regarded as rare, and is poorly defined. Ilan et al. [31] recently demonstrated that E. coli possessed a gene that encoded a protein tyrosine kinase. The issue of whether or not S. aureus has a PTK gene, which might represent a target for genistein, is presently unresolved. Although daidzein lacks PTK-inhibitory properties, it inhibited staphylococcal growth, although to a lesser degree than genistein. Thus, the mechanism by which genistein inhibits bacterial growth is probably not at the level of PTK inhibition.

We also considered that genistein might exert bactericidal activities by modulating the methylation patterns of the bacterial DNA. Indeed, bacterial DNA methylation has been demonstrated to affect bacterial cell cycling [32], and it has been shown that genistein alters the DNA methylation patterns in eukaryotic cells [33]. The mechanism underlying the anti-staphylococcal activity by genistein does not appear to be mediated by changes in DNA methylation, since there were no apparent differences in the HpaII (methylation sensitive) and MspI (methylation resistant) restriction enzyme digestion patterns of the genomic DNA between genistein-treated and untreated bacteria. Furthermore, an E. coli strain exhibited slowing of replication time and lengthened generation times when expressing the murine DNA maintenance methyltransferase [34]. Since the growth of E. coli was not inhibited by genistein, it seems likely that the growth-inhibiting effects of genistein on the other bacterial strains does not depend on the DNA methylation patterns.

Genistein had no major impact on the development of arthritis in mice that were inoculated with S. aureus. Mice that were treated with 60 mg of genistein per kg body weight exhibited attenuated severity and frequency of arthritis on days 5, 7 and 9 post-infection compared with the controls. Thus, it appears that genistein treatment alters the course of

Fig. 5. Severity (A) and frequency (B) of arthritis in mice (n = 10 in each group) treated with 60 mg of genistein or vehicle solution per kg body weight every second day, starting 2 days prior to i.v. inoculation with S. aureus LS-1.
septic arthritis development. However, no differences were observed between the groups in terms of the numbers of bacteria in the blood or kidneys. The decrease in clinical signs of arthritis in the genistein-treated animals may be ascribed to the ability of genistein to reduce fluid retention and capillary permeability [35]. The immunosuppressive and antibacterial effects of genistein may be synergistic. Previously, we demonstrated that genistein had anti-inflammatory properties [21]. Other studies have shown that the migration of inflammatory cells is downregulated by genistein, since leukocyte adherence to endothelial cells is attenuated by genistein [36,37]. In addition, genistein inhibits the release of inflammatory substances by activated macrophages [38] and neutrophils [39], thereby contributing to the downregulation of inflammatory responses.

In summary, this study demonstrates the in vitro growth-inhibitory effect of genistein on various bacteria, including methicillin-resistant strains of *S. aureus*. Therefore, genistein may represent a new type of anti-staphylococcal agent, whose activity appears to involve the stabilization of the covalent topoisomerase II-DNA cleavage complex.

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