

Green tea polyphenols reduce autoimmune symptoms in a murine model for human Sjogren's syndrome and protect human salivary acinar cells from TNF- α -induced cytotoxicity*

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(Submitted: 3 August 2006; accepted with revisions: 8 December 2006)

Abstract

Sjogren's syndrome (SS) is a relatively common autoimmune disorder. A key feature of SS is lymphocytic infiltration of the salivary and lacrimal glands, associated with the destruction of secretory functions of these glands. Current treatment of SS targets the symptoms but is unable to reduce or prevent the damage to the glands. We reported previously that the major green tea polyphenol (GTP) epigallocatechin-3-gallate (EGCG) inhibits autoantigen expression in normal human keratinocytes and immortalized normal human salivary acinar cells (Hsu et al. 2005). However, it is not known whether GTPs have this effect *in vivo*, if they can reduce lymphocytic infiltration, or protect salivary acinar cells from tumor necrosis factor- α (TNF- α)-induced cytotoxicity. Here, we demonstrate that in the NOD mouse, a model for human SS, oral administration of green tea extract reduced the serum total autoantibody levels and the autoimmune-induced lymphocytic infiltration of the submandibular glands. Further, we show that EGCG protected normal human salivary acinar cells from TNF- α -induced cytotoxicity. This protection was associated with specific phosphorylation of p38 MAPK, and inhibitors of the p38 MAPK pathway blocked the protective effect. In conclusion, GTPs may provide a degree of protection against autoimmune-induced tissue damage in SS, mediated in part through activation of MAPK elements.

Keywords: Green tea polyphenols, EGCG, Sjogren's syndrome, acinar cells, MAPK

Introduction

Sjögren's syndrome (SS) is an autoimmune disorder that can affect multiple organs. Primary SS is associated with lymphocytic infiltrations of the salivary and lacrimal glands and eventual atrophy, leading to a loss of fluid production. The salivary component of SS is defined as xerostomia, with symptoms generally referred to as salivary hypofunction. If not treated, xerostomia may lead to oral complications [1]. Estimates of the prevalence of SS are affected by the criteria used for diagnosis.

However, genuine differences between various regions and communities exist [2,3]. The world-wide distribution is believed to be 1/2500 [4]. In the US, SS affects approximately 1% of the population [5]. In China, one regional study with 26,000 subjects suggested the prevalence of primary SS was only 0.03% [6]. In Japan, the estimated crude prevalence rates for SS were only 1.9 and 25.6 per 100,000 population in males and females, respectively [7]. A survey conducted by the Japanese Ministry of Health and Welfare indicated the SS prevalence was just 0.06% among females [8]. As for xerostomia, one

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*This study was supported in part by funds from NIH grant CA097258, and Department of Oral Biology and Maxillofacial Pathology, School of Dentistry to S.H. Data in this manuscript was presented in poster form at the annual meeting of the American Association for Dental Research, 2006.

study showed that among a group of 1003 Japanese individuals with an average age of 66, about 9.1% experienced dry mouth during eating [9], whereas in the US, one epidemiological study found that in a group of 2481 individuals aged 65–84 years old, 27% reported either dry mouth or dry eyes [10], and another found that dry mouth ranged from 10% among persons over age 50–40% for persons over age 65 [11]. Although precise statistical comparison between the US population and either the Japanese or Chinese population is not available, it is apparent that SS and xerostomia are more prevalent in the US population, particularly amongst the elderly.

SS is not a curable or preventable disease at present, and whether it can be prevented or delayed is unknown. Treatment is generally symptomatic and supportive (National Institute of Arthritis and Musculoskeletal and Skin Diseases). For xerostomia and xerophthalmia, artificial lubricants are commonly used as saliva or tear substitutes [12]. In recent years, salivary stimulants, such as pilocarpine and cevimeline, have been approved by the FDA to treat xerostomia [13]. In addition, oral administration of interferon γ (IFN- γ) was effective in improving saliva production in patients with primary SS [14]. However, long-term adverse effects have not been evaluated for these therapies. Gene therapy might be one of the future treatments for primary SS by inducing the growth and differentiation of glands [13]. On another front, herbal extracts and Chinese traditional medicine have been used to treat SS and/or xerostomia with a certain degree of success [15,16]. These naturally occurring materials may provide an alternative remedy for SS-associated disorders. One group of potentially useful phytochemicals is green tea polyphenols (GTPs).

GTPs, also referred to as green tea catechins, are a group of polyphenolic compounds present in the leaves of *Camellia sinensis*. The major GTPs are (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and (–)-epigallocatechin-3-gallate (EGCG). EGCG is the most abundant and widely studied of these compounds [17–19]. Work during the past 20 years has demonstrated that GTPs are potent antioxidants that also possess chemopreventive, anti-apoptotic, and anti-inflammatory activities. These properties are consistent with an apparent beneficial effect of GTPs against a range of diseases [20,21]. Consumption of green tea is high in China and Japan, regions where the incidence of SS and xerostomia are substantially lower than the US [5–10].

Apoptosis, autoantigen expression and cytotoxicity are major cell-based mechanisms that have been implicated in SS pathogenesis [22]. We reported previously that EGCG exposure resulted in a significant reduction of expression of several autoantigens (including SS-A, SS-B, fodrin, centromere protein C, golgin-67, coilin, and PARP) by various

cell types [23]. Further, we have shown that EGCG suppressed reactive oxygen species (ROS) levels in human salivary gland acinar cell-derived NS–SV–AC cells in a dose-independent manner [24]. Tumor necrosis factor- α (TNF- α), which can be produced by inflammatory cells, is known to induce cytotoxicity in many cell types. One of the genes previously shown to be down-regulated by EGCG was TNF- α [25,26]. Studies using normal human keratinocytes have shown that EGCG modulates signaling via the p38 MAPK pathway, downstream of MEK3 [27]. Since EGCG in the effective 15–100 μ M range is physiologically achievable via oral consumption [28], we hypothesized that oral administration of GTPs could protect the salivary glands from SS-induced tissue destruction by attenuating one or more cell-based mechanisms of pathogenesis (apoptosis, autoantigen gene expression and/or cytokine production) via two molecular mechanisms—antioxidant activity and p38 activation in acinar cells [22]. The non-obese diabetic (NOD) mouse is an important model system that has provided clues to the cellular mechanisms involved in SS [29]. This mouse strain develops a lymphocytic infiltration of exocrine tissues at 10–12 weeks of age, particularly in females, and was originally used as a model for type I diabetes. The current study provides evidence for a protective role of GTPs in NOD mice, and demonstrates that protection of acinar cells from TNF- α mediated cytotoxicity *in vitro* involves p38 signaling.

Materials and methods

Chemicals and antibodies

EGCG was purchased from Sigma-Aldrich (St Louis, MO). Anti-human p38, pp38, pJNK and pERK antibodies, and anti-human actin (I-19) goat polyclonal antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The Mouse Anti-Nuclear Antibodies (ANA) ELISA Kit was purchased from Alpha Diagnostic International, Inc. San Antonio, TX. Specific inhibitors for p38 (SB 203580), JNK (SP600125) and ERK (PD 98059) were supplied by EMD Bioscience, Inc., San Diego, CA. The green tea extract with 70% GTPs (referred as to GTPs hereafter) are provided by Zhejinag Cereals, Oils & Foodstuffs Imp/Exp Co., Ltd, China, which contain 40% EGCG, 13% ECG, 7.3% EGC, 3.2% EC, and 2.7% Caffeine. The rationale to use this green tea extract instead of purified GTPs is to mimic green tea oral consumption to evaluate the *in vivo* effects.

Animal treatment

The NOD mice were purchased from Jackson Laboratory. All animal protocols in this study were approved by the Institutional Animal Care and Use Committee. Animals (two groups, 15 NOD

mice/group) were allowed *ad libitum* access to either water or 0.2% GTPs starting at the 9th week of age. After the onset of autoimmune disease (determined as diabetes, detected by Glucotest strips), each animal was allowed to progress with the disease for 3 weeks, and then euthanized (2 water-fed control mice died during this 3-week period). Blood was collected from each animal in the above described treatment groups by cardiac puncture immediately after euthanasia, and serum for ELISA assays was prepared by centrifugation of blood samples at 3000 rpm. The submandibular glands were dissected free of the sublingual gland and attached tissues for pathological analyses.

Determination of serum total autoantibodies. Samples from the two groups were examined by ELISA assays for anti-SS-associated autoantibodies using the Mouse Anti-Nuclear Antibodies (ANA) ELISA Kit (Cat #5200, Alpha Diagnostic International, Inc. San Antonio, TX) according to the manufacturer's instructions. This kit detects total ANA against ds-DNA, ss-DNA, histones, ribonucleoproteins (RNPs), SS-A, SS-B, SM antigens, Jo-1, and Scl-70. Samples in triplicate were analyzed with blanks, positive and negative controls in 96-well plates by ELISA reaction, photo-detection using a VERSAmax Microplate Reader at 450 nm, and statistical analysis using two-tailed student *t*-test.

Tissue processing

The submandibular glands from NOD mice were fixed in 10% neutral-buffered formalin, paraffin embedded, sectioned at 5 μ m, and stained with H&E by routine methods previously described [23].

Pathology scoring of lymphocyte infiltration in the submandibular glands of NOD mice

We adopted the cumulative focus score (cFS) criteria recently published by Morbini et al. [30] for the assessment of salivary gland inflammatory infiltrates as a component of the diagnosis of Sjogren's syndrome (SS). Briefly, these criteria modify the American-European Consensus Group [3] focus score (FS) criterion as a component of the diagnosis of SS by adopting a multilevel sectioning and evaluation of salivary gland tissues of suspected SS patients. This multilevel sampling improved the diagnostic accuracy of biopsies with a baseline FS between 1 and 2, which represents the critical cutoff in SS histopathological evaluation [30]. The cFS method assesses the number of chronic (lymphocytic) inflammatory cell infiltrates of at least 50 in a 10-HP (equivalent to 4 mm²) light microscopy field repeated for a minimum of three different tissue section levels. The arithmetic average FS from all examined levels from a particular gland represents the cFS average

for that gland. To examine differences in the size of foci above 50 cells, a quantitative analysis was performed using the BIOQUANT NOVA PRIME 6.75 software. One H&E-stained submandibular salivary gland section was selected at random for each of the animals in the two groups and images of areas containing foci were loaded into the software. The areas of lymphocyte infiltration foci were captured individually and measured quantitatively by the software as relative density units.

Cell line

The immortalized human salivary gland acinar cells (NS-SV-AC) have been described previously [31]. These cells were selected following transfection of origin-defective SV40 mutant DNA and maintained in keratinocytes growth medium-2 (KGM-2, Cambrex, East Rutherford, NJ). They were kindly provided by Dr Masayuki Azuma (Tokushima University School of Dentistry, Tokushima, Japan). Subculture of the cells was performed by detaching the cells in 0.25% trypsin, and transferring into new tissue culture flasks.

Cell treatment

Since EGCG is the most abundant green tea constituent, whether EGCG possesses protective effects to human salivary acinar cells against TNF- α , and through which signaling pathway EGCG acts upon are specific issues to be addressed. EGCG was dissolved in cell culture medium as a 50 mM stock immediately before use. To assess the possible protective effect of EGCG against TNF- α -induced cytotoxicity, MTT assays were performed (described in sections below). To determine which specific MAPK pathways is essential for EGCG signaling, exponentially growing NS-SV-AC cells were incubated with 50 μ M EGCG for various time periods in the growth medium described above, harvested at same time (thus, all cells were cultured in identical conditions except exposure time to EGCG), and then were extracted for cell lysates prior to Western blots.

Western blot

Cells were washed in ice-cold PBS and lysed for 20 min in RIPA buffer containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 μ g/ml leupeptin, 3 μ g/ml aprotinin and 100 mM phenylmethylsulfonyl fluoride (PMSF). Samples of lysates containing the same amount of protein were loaded in each lane (we used 5–30 μ g, depending on the antibody used) and electrophoretically separated on a 12% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to a PVDF membrane (Immobilon™-P, Millipore Corporation,

Bedford, MA). The membrane was blocked for 1 h with 5% (w/v) non-fat dry milk powder in PBST (0.1% Tween-20 in PBS) and then incubated for 1 h with primary antibody diluted in PBST/milk (antibodies and dilutions: rabbit polyclonal JNK1/2, 1:1000, mouse monoclonal pJNK1/2, 1:1000, rabbit polyclonal ERK, 1:2000, rabbit polyclonal p38, 1:1000, rabbit polyclonal pp38, 1:1000 and goat polyclonal actin, 1:2000). The membrane was washed three times with PBST and incubated with peroxidase-conjugated, affinity-purified anti-rabbit IgG (Santa Cruz Biotechnology Inc.) for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL Western blotting detection reagents (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Experiments were repeated three times with similar results and identical patterns.

TNF- α -induced cytotoxicity

To determine the possible protective effect of EGCG, NS-SV-AC cells (0.5×10^4 cells/well) were seeded in a 96-well microplate in triplicates and incubated overnight prior to either the addition of EGCG at 0, 3, 6, 12.5, 25 and 50 μ M alone, or in combination with 100 ng/ml TNF- α and 10 μ g/ml cycloheximide. To determine if p38 MAPK inhibitor blocks the protective effect of EGCG, p38 MAPK and MEK (upstream to p38 MAPK) inhibitors were incubated with NS-SV-AC cells, in triplicates, for 30 min at 0, 10, 20 and 30 μ M prior to TNF- α /cycloheximide and EGCG (50 μ M) treatment as described above. These inhibitors were also tested at 30 μ M, either with or without the presence of TNF- α /cycloheximide, to determine whether any of the inhibitors influenced the cell viability or cytotoxicity induced by TNF- α . After the treatments, viability was determined by the MTT assay. The cells in each well were washed with 200 μ l of phosphate-buffered saline (PBS) and incubated with 100 μ l of 2% (w/v) MTT in a solution of 0.05 M Tris-HCl (pH 7.6), 0.5 mM MgCl₂, 2.5 mM CoCl₂ and 0.25 M disodium succinate at 37°C for 30 min. Cells were fixed by the addition of 100 μ l of 4% (v/v) formalin in 0.2 M Tris-HCl (pH 7.6), and after a 5 min incubation at room temperature liquid was removed and the wells were allowed to dry. Each well was rinsed with 200 μ l water and cells in each well were solubilized by the addition of 100 μ l of 6.35% (v/v) 0.1 N NaOH in DMSO. The colored formazan product was measured by a Thermo MAX micro plate reader (Molecular Devices Corp. Sunnyvale, CA) at a wavelength of 562 nm.

Statistical analysis

All data are reported as mean \pm SD or mean \pm SEM. A one-way ANOVA and two-tailed Student's *t*-tests

were used to analyze statistical significant. Differences were considered statistically significant at $p < 0.05$.

Results

GTPs reduced serum autoantibody levels in NOD mice

NOD mice were fed either water or water containing 0.2% GTPs for 3 weeks. Sera from 27 animals were analyzed for total autoantibody levels by ELISA, 15 animals from the GTP-water and 12 from the water-only group (in the latter group, two animals died before the end of 3-week disease progression, and insufficient serum was collected from another). There was a significant difference between the total serum antibody levels from the GTP-water group and water-only group. On average, the total ANA (against ds-DNA, ss-DNA, histones, RNPs, SS-A, SS-B, SM antigens, Jo-1, and Scl-70) in the GTP-water animals was approximately 20% lower than that of the water-only animals (Figure 1). This result indicates that oral administration of GTPs significantly reduced the serum autoantibody levels (two-tailed student *t*-test, $p = 0.036$, $n = 27$).

GTPs reduced lymphocyte infiltration in NOD mouse submandibular glands

The submandibular glands of each NOD animal were collected and the standardized scores for the inflammatory cell infiltrates were determined blindly, as described in the methods. Figure 2 shows representative submandibular glands from a water-fed control (A) and a GTP/water-fed NOD mouse (B). Pathological

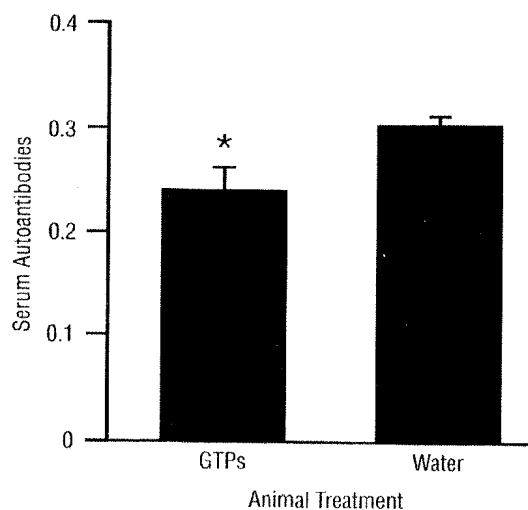


Figure 1. Total serum autoantibody determination by ELISA. Serum autoantibody concentration in NOD mice after 3 week-progression of autoimmune disease in each mouse was measured by the mouse anti-nuclear antibodies (ANA) ELISA Kit. Values of each bar represent optic absorption measurements for each group. y -error bars represent SEM. Two-tailed student *t*-test analysis, $p = 0.036$, $n = 27$.

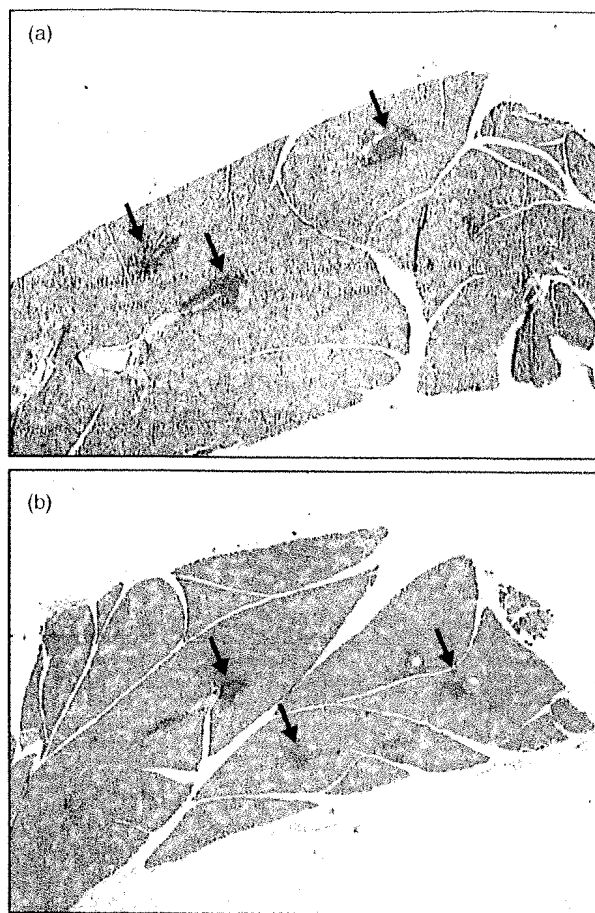


Figure 2. Representative H&E stained sub-mandibular gland sections. Glands were collected from (A) water-only, and (B) GTPs-water treated NOD mice after autoimmune disease progressed 3 weeks in each animal. Arrows point to local lymphocytic infiltrations consisting 50 or more lymphocytes.

focal scoring, using the cFS criteria for human SS diagnosis, demonstrated no significant difference in focal scores (i.e. the number of focal inflammatory cell aggregates containing 50 or more lymphocytes in each 4 mm^2 area) between the GTP-treated and untreated (water) controls. The average focal score was 2.125 ± 1.13 for GTP-fed mice and 2.125 ± 0.64 for control mice. However, inspection of the foci suggested potential differences in the focal areas between the groups, equivalent to differences in the total number of inflammatory cells/focus. For example, both animals shown in Figure 2 received a focal score of 3, but the foci in the GTP-treated animal appear smaller (although for any one focal group this might just reflect an off-center cut through its volume). Quantitative analysis of the areas of lymphocyte infiltration foci in H&E-stained submandibular gland sections (Figure 3, $n = 27$ animals) demonstrated an approximately 30% difference between the groups in the number of inflammatory cells/infiltrate, with fewer cells in the salivary glands of GTPs/water-fed animals ($p = 0.006$, two-tailed t -test, $n = 83$ foci/group).

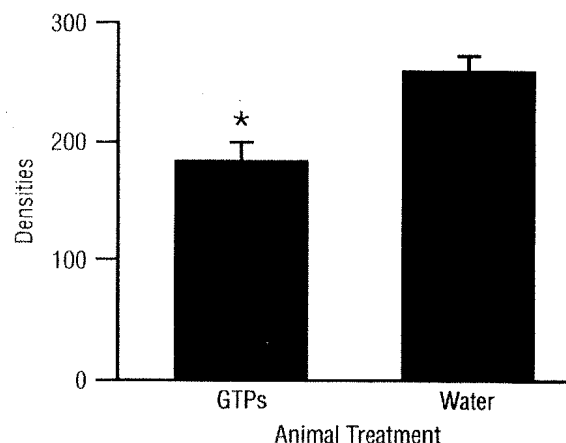


Figure 3. Average focal areas of submandibular lymphocyte infiltration in NOD mice. Animals were fed either with GTPs/water or water only, starting at week nine, and the autoimmune disease was allowed to progress for 3 weeks. Relative density units were generated by BIOQUANT NOVA PRIME 6.75 software, representing area sizes. Error bars are standard error of means (SEM). Results were analyzed by two-tailed t -test analysis ($p = 0.006$). $n = 83$ foci/group.

Human salivary gland acinar cells are protected from TNF- α -induced cytotoxicity by EGCG

TNF- α , which is produced by inflammatory cells, is known to induce cytotoxicity in many cell types, and may contribute to SS [32–34]. Its production can be down-regulated by EGCG [25,26]. We examined the effects of TNF- α on the human salivary gland cell line NS–SV–AC using the MTT viability assay. TNF- α at a concentration of 100 ng/ml in the presence of cycloheximide reduced the viability of NS–SV–AC cells to approximately 30% of untreated control cells (Figure 4).

A second mechanism by which EGCG could ameliorate the effects of TNF- α is by attenuation of the cytotoxic effect at the target acinar cells. As determined by the MTT assay, simultaneous treatment with EGCG and TNF- α partially protected the cells, with an improved viability of 50% of control (two-tailed t -test, $p < 0.05$, $n = 3$). EGCG at these concentrations in the absence of TNF- α had no effect on the viability of the cells ([35], and data not shown).

EGCG specifically activates the phosphorylation of p38 in NS–SV–AC cells

Activation of p38 by phosphorylation can be protective [36]. We hypothesized that p38 phosphorylation in acinar cells could be one of the mechanisms by which GTPs attenuate SS pathogenesis. As shown in Figure 5(A), EGCG induced a rapid and sustained phosphorylation of p38 in NS–SV–AC cells.

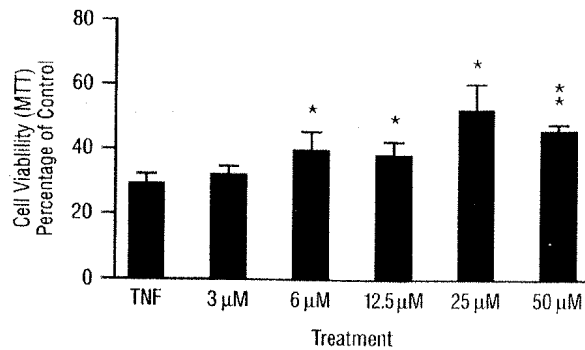


Figure 4. Cell viability assay (MTT) result of NS-SV-AC cells treated with TNF- α with or without increasing concentrations of EGCG. Cells were treated with the indicated concentrations of EGCG in addition to 100 ng/ml TNF- α and 10 μ g/ml cycloheximide (TNF). Cells without incubation with EGCG exhibit less than 30% of viability compared to control without TNF- α or EGCG. The cell viability increased to nearly 40% of the control levels in medium contained 12 μ M EGCG. Cell viability further increased to approximately 50% of control level in medium contained 25 or 50 μ M EGCG. Data represent one of two triplicate experiments with similar patterns. * Significantly different than the control value ($p < 0.05$, $n = 3$). **Significantly different than all other values ($p < 0.05$, $n = 3$).

In contrast, EGCG induced only a slight increase in pERK levels, and had no effect on phosphorylation of JNK.

TNF- α activates JNK and p38 phosphorylation in NS-SV-AC cells

TNF- α has been shown to induce phosphorylation of JNK, leading to cytotoxicity and apoptosis in various cells. When NS-SV-AC cells were exposed to TNF- α , a rapid and prolonged phosphorylation of JNK was observed (Figure 5(B)). Interestingly, p38 phosphorylation was also induced with similar kinetics to those seen for JNK. ERK phosphorylation (pERK)

was not affected significantly. Simultaneous exposure to EGCG had no effect on TNF- α induced phosphorylation of JNK or p38, and pERK remained unchanged (Figure 5(C)).

p38 MAPK inhibitor SB203580 and MEK inhibitor PD98059 abolished the protective effect of EGCG

To further examine the effects of EGCG on MAPK signaling pathway activation and TNF- α induced cytotoxicity, cells were exposed to combinations of TNF- α , EGCG, and either the p38 MAPK inhibitor SB203580 or the MEK inhibitor PD98059. The MEK inhibitor blocks the activation of both p38 and pERK. Although JNK-specific inhibitors are available, we have found they are highly toxic to NS-SV-AC cells, presumably due to a requirement for a low level of pJNK [37,38].

Both inhibitors tested abolished the protective effects of EGCG on TNF- α induced cytotoxicity (two-tailed t -test, $p < 0.05$, $n = 3$) (Figure 6). The viability levels with TNF- α + inhibitor + EGCG were approximately 30% of control (untreated cells or cells treated with EGCG alone), similar to the viability seen with TNF- α alone (Figure 6). That is, inhibition of the p38 signaling pathway eliminated the protective effects of EGCG. The inhibitors alone had no effect on the viability of the cells (data not shown).

Discussion

SS is an autoimmune disorder characterized by inflammatory cell infiltration of the salivary and lacrimal glands, destruction of secretory acinar cells, and salivary or lacrimal hypofunction. The potentially beneficial effects of GTPs on SS have not been explored previously. Several lines of published evidence suggest GTPs have a beneficial effect in

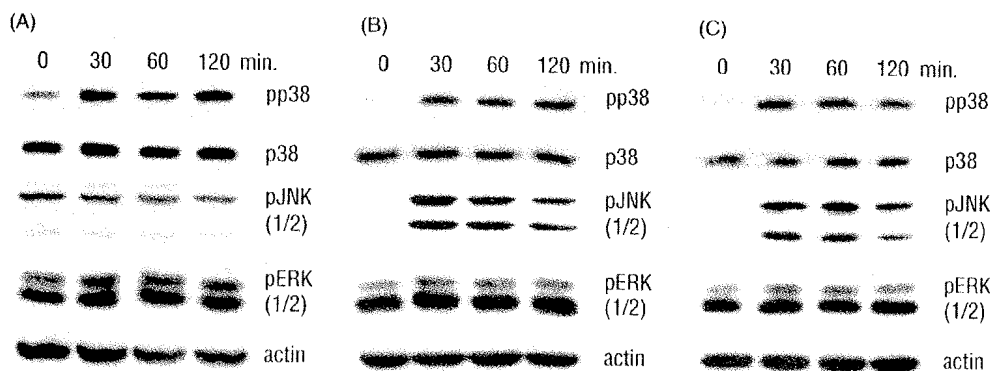


Figure 5. Phosphorylation of p38, JNK and ERK at 0, 0.5, 1 and 2 h in NS-SV-AC cells. (A) Phosphorylation of p38 (pp38) was induced significantly by 50 μ M EGCG at 30 min and remained elevated through out the 120 min period. Levels of p38 protein did not change. EGCG failed to alter JNK phosphorylation (pJNK) while its modestly increased pERK. (B) TNF- α alone induced a rapid and prolonged phosphorylation of both p38 and JNK proteins, but failed to modulate the phosphorylation of ERK proteins. (C) Simultaneous addition of EGCG and TNF- α induced a rapid and prolonged phosphorylation of both p38 and JNK proteins. (1/2): JNK/ERK 1 and 2. The pJNK panel in Figure 5(A) was exposed 10 times longer than the corresponding panels in Figure 5(B),(C).

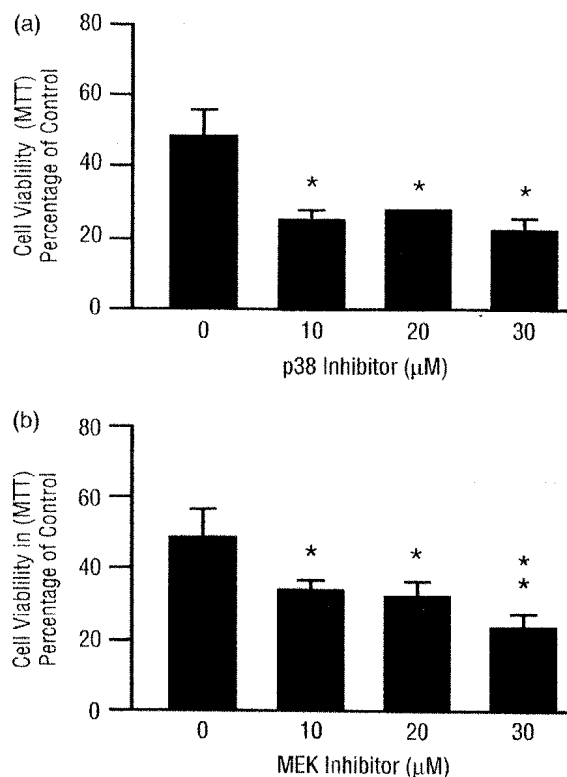


Figure 6. Cell viability assay (MTT) result of NS-SV-AC cells treated with TNF- α , 25 μ M EGCG, p38 or MEK inhibitor. (A) p38 inhibitor at 10–30 μ M abolished EGCG effect. (B) MEK inhibitor at 10–30 μ M dose-dependently abolished EGCG effect. 0: TNF- α plus EGCG without inhibitors; 10–30: TNF- α plus EGCG and inhibitors with increasing concentrations. Control samples were treated with EGCG only as 100%. *Significantly different than inhibitor-free value ($p < 0.05$, $n = 3$). **Significantly different than all other values ($p < 0.05$, $n = 3$).

individuals with autoimmune disorders. Recent studies of the anti-inflammatory effects of green tea have shown promising results [39]. An epidemiological study of 31,336 females, aged 55–69 years, and conducted from 1986 to 1997, concluded that there was an inverse association between tea consumption and the onset of rheumatoid arthritis (RA). The relative risk (RR) of RA decreased to 0.39 (RR 0.39, 95% CI 0.16–0.97) in women consuming more than 3 cups of tea compared to women who never consumed tea [40]. Previously reported animal studies using autoimmune mouse models suggested that consumption of green tea or GTPs could reduce certain autoimmune symptoms. In a mouse model for RA, oral administration of 0.2% of GTPs significantly reduced the protein levels of COX-2, IFN- γ , TNF- α and IgG in the joints, and resulted in a more than 50% reduction of collagen-induced arthritis [41]. Similarly, in a mouse model for autoimmune encephalomyelitis, oral administration of EGCG reduced clinical severity when the compound was given at initiation or after the onset of the symptom, both by limiting brain

inflammation and by reducing neuronal damage [42]. These effects were accompanied by abrogated proliferation and TNF- α production in encephalitogenic T cells [42]. Animal studies using transgenic mice demonstrated that transcription of TNF- α and IL-6, cytokines associated with inflammation, was inhibited by 0.1% green tea extract in drinking water. This evidence suggests that green tea may moderate or prevent diseases associated with cytokine overexpression [19]. Both *in vitro* and *in vivo* studies demonstrated that EGCG effectively protected animals from MLD-STZ-induced diabetes (a model for type I diabetes) by protecting pancreatic islets, suggesting a possible therapeutic value of EGCG for the prevention of diabetes mellitus [43]. In a lupus erythematosus (LE) mouse model MRL/MpJ-Fas^{lpr}/Fas^{lpr} (MRL-lpr^{CG}), mice were fed with a 2% green tea powder diet for 3 months. The green tea-fed group showed a prolonged survival period, with reduced lymph node hyperplasia and reduced levels of anti-DNA antibodies [44]. Taken together, these data show that GTPs can reduce the severity of autoimmune diseases. One mechanism could be a protective response in target cells activated by GTPs/EGCG, possibly through modulation of the p38 MAPK pathway. The current study focused on the effect of oral consumption of GTPs on lymphocyte infiltration in the salivary glands, the possible signal pathway(s) activated by EGCG in human salivary acinar cells, and interactions of EGCG with TNF- α -induced cytotoxicity. Our overall hypothesis is that oral administration of GTPs could protect the salivary gland from SS-induced tissue destruction by attenuating one or more cell-based mechanisms of pathogenesis (apoptosis, autoantigen gene expression and/or cytokine production) via two molecular mechanisms—p38 activation and antioxidant activity in acinar cells (Figure 7).

Results from the current study using the NOD mouse as a model for SS demonstrated a significant reduction of total serum autoantibody levels in GTP-treated animals, compared with the untreated control NOD animals (Figure 1). Similarly, the size of lymphocytic infiltrate foci was also reduced after GTP treatment (Figures 2 and 3). These data indicate that GTPs have a protective effect against autoimmune responses in the NOD mouse. Green tea consumption by humans leads to an increase of secreted salivary GTPs, in a concentration range (50 + μ M) 10 times higher than the serum levels [28]. We note that the GTP-treated group had an average 1 week delay in the onset of autoimmune diabetes (data not shown), and while all of the 15 GTP-fed NOD mice survived the 3-week disease progression period, two untreated control mice died during this period. Although these latter observations do not reach statistical significance due to the sample size, the trend is consistent with a GTP-mediated amelioration

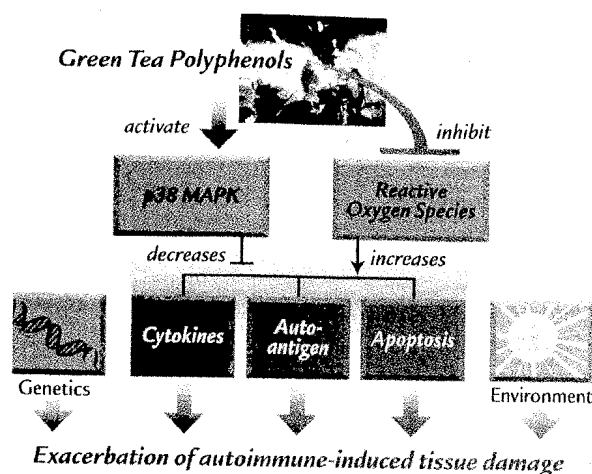


Figure 7. Schematic model for GTP-mediated protective mechanism. Observations on the protective role of GTPs against the damage incurred by autoimmune disorders suggest that three potential major strategies for ameliorating SS directly involving the acinar cells could be selective inhibition of (i) their apoptosis, (ii) autoantigen expression, and (iii) production of pro-inflammatory cytokines. Inhibition could be achieved by activation of the p38 MAPK pathway or reduction in reactive oxygen species, which would reduce JNK activation.

of autoimmune pathology, and warrant further exploration.

Although the size of the foci showed a significant difference between the two groups, the focal scores based on human diagnostic criteria did not differ. This could be due to species differences or more subtle differences between human SS and the NOD mouse model. A further possibility is that the time of onset of GTP treatment (9 weeks of age) might be relatively late with respect to the initial phases of the process of gland damage and initiation of focus formation. NOD-scid congenic mice (that lack functional lymphocytes) do not develop sialadenitis (or diabetes). However, they do show dysfunction in expression of biochemical markers of salivary gland differentiation such as amylase and parotid secretory protein (PSP). These data are consistent with a model for SS in which there is an initial phase, during which dysregulation of glandular homeostasis triggers the disease, followed by an immune cell-mediated phase that leads to a loss of secretory function [29]. We are currently examining the effects of timing of EGCG exposure.

The multiple MAPK signal transduction pathways are involved in the control of diverse cellular events including proliferation, differentiation and apoptosis. Gene expression in salivary epithelial cells is regulated, in part, via the Raf/MEK/MAPK pathway [45]. It was found that Raf-1 kinase-induced down-regulation of a sodium channel was blocked by the MEK inhibitor PD98059, suggesting that the ERK pathway is involved in the signal transduction [46]. The acinar

cells respond to nitric oxide (NO), an inflammation-related signaling molecule, by the pathways regulated by ERK and p38 [47]. The p38 MAPK pathway is important in transducing stress signals, and p38 MAPK is strongly and rapidly activated by stresses and inflammatory cytokines. Recently, it was suggested that inhibition of LPS-stimulated iNOS and COX-2 expression and reduced NO release were by a mechanism involving p38 [48]. SS patients show activated forms of p38 and JNK in infiltrating mononuclear cells [49]. The p38 MAPK family consists of at least four isoforms. The specificity of the isoforms activated depends on the cell type, and the nature and strength of the signals [50]. Protein kinases downstream of p38 can activate transcription factors such as activating transcription factor-2 (ATF-2) and growth and DNA damage (GADD)-153 transcription factor. Importantly, the cellular response to p38 MAPK activation is highly cell type dependent: it can induce apoptosis, growth arrest, or differentiation [39,44]. It is known that GTPs modulate the MAPK pathways [51]. Here, we show that, in the human acinar cell line NS-SV-AC, EGCG selectively induced the activation of p38 by phosphorylation: p38 is rapidly and specifically phosphorylated within 30 min following EGCG exposure, while levels of pJNK and pERK were relatively unchanged (Figure 5(A)). Thus, EGCG can activate the p38 signaling pathway, and may therefore lead to activation of a protective response within acinar cells, and/or beneficial modulation of cytokine signaling within inflammatory foci.

The survival of a cell is the outcome of the position established between two opposing systems: pro- and anti-cell death pathways. TNF- α is a multifunctional cytokine that can activate several pathways in cells following binding to the TNF-R1 receptor [52]. Ligand binding allows formation of two complexes with the receptor: Complex I leads to activation of NF κ B that in turn activates antiapoptotic genes, including various antioxidant enzymes; Complex II is involved in activation of caspases 8 and 10, leading to apoptosis. TNF- α also induces accumulation of ROS, although the mechanism is not fully understood [53]. The JNK MAPK signaling pathway can be activated by ROS, providing a third route to cell death via activation of the mitochondria-dependent pathway [53]. NF- κ B can downregulate TNF- α -induced JNK activation, at least in part via upregulation of antioxidant enzymes. Thus, there is complex crosstalk between the various pro- and anti-apoptotic pathways activated by TNF- α , and ROS play an important role in this signaling. Inhibition of protein synthesis (e.g. by cycloheximide) suppresses the antiapoptotic pathways, and TNF- α is then a potent inducer of cell death in many different lines. Under these conditions, TNF- α induced marked cytotoxicity in NS-SV-AC cells, reducing cell viability by about 70%. Consistent with the pathways outlined

above, TNF- α also rapidly induced JNK phosphorylation (pJNK) in these cells (Figure 5(B)). Although TNF- α induced p38 phosphorylation in these cells (Figure 5(B)), pro-death effects outweighed any protective benefit. In contrast, NS-SV-AC cells exposed to TNF- α in the presence of physiological levels of EGCG showed considerably less cell death as measured by the MTT assay. This was not due to immediate enhanced p38 phosphorylation, since the pp38 levels are comparable between TNF- α alone and TNF- α + EGCG during the 2 h period examined (Figure 5(C)). We are examining the possibility that differences emerge over a longer timeframe.

We have previously reported that EGCG is a potent inhibitor of ROS in NS-SV-AC [24]. This provides a plausible explanation for the beneficial effects of EGCG seen here in TNF- α -induced cytotoxicity. A reduction in the level of TNF- α induced ROS would have the effect of reducing JNK activation, in turn reducing pro-apoptotic signaling, and increasing survival. However, this survival is dependent upon a balancing p38 phosphorylation, since inhibition of p38 or MEK (a MAPK upstream of p38) eliminates the protective effect of EGCG (Figure 6(A),(B)). Under these conditions, TNF- α could still activate caspases 8 and 10, leading to apoptosis.

In conclusion, in the NOD mouse model of SS, oral administration of GTPs lowered the total serum autoantibody level and reduced the magnitude of salivary lymphocyte infiltration. *In vitro*, EGCG partially protected human acinar-derived cells from TNF- α -induced cytotoxicity, and this protective effect of EGCG involves indirectly the p38 MAPK-signaling pathway. The *in vivo* studies in this report examined the effects of GTPs after the onset of overt autoimmune disease. It is possible that GTPs may be able to play a role in the prevention of glandular damage by activation of protective systems early in disease onset through both gene regulation via p38 MAPK pathway and antioxidant activities, as illustrated in Figure 7. Future investigation is needed to test this hypothesis. We anticipate that GTPs could ultimately be used as an alternative approach to prevent and manage certain autoimmune diseases.

Acknowledgements

The authors thank Dr Robert Podolsky for statistical assistance and Laura McKie for graphical work. This study was supported in part by funds from NIH grant CA097258-01A1, and Department of Oral Biology and Maxillofacial Pathology, School of Dentistry to S.H.

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