Resveratrol Alters Proliferative Responses and Apoptosis in Human Activated B Lymphocytes in Vitro\textsuperscript{1,2}

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Abstract

We hypothesized that the phytochemicals resveratrol, quercetin, and kaempferol would modulate B lymphocyte proliferation, Ig synthesis, and apoptosis after activation. Peripheral blood mononuclear cells (PBMC) were isolated from 12 healthy adult human volunteers and incubated with pokeweed mitogen plus 0, 2, 5, and 10 \(\mu\)mol/L resveratrol, quercetin, or kaempferol. After 6 d, CD19\(^+\) B cells were analyzed for proliferation, B cell lymphoma-2 (Bcl-2) expression, and activation of caspase-3 using flow cytometry. After 8 d, cell supernatants were collected and IgM and IgG were measured by ELISA. Resveratrol at a concentration of 5 \(\mu\)mol/L increased the percentage of CD19\(^+\) cells compared with mitogen only-stimulated cells (\(P < 0.01\)), and a trend for increased proliferation was observed for cells treated with 0, 2, and 5 \(\mu\)mol/L resveratrol (\(P\)-trend = 0.01). However, 10 \(\mu\)mol/L resveratrol inhibited proliferation of B lymphocytes (\(P < 0.01\)). Expression of Bcl-2 and caspase-3 activation increased in B cells treated with 10 \(\mu\)mol/L resveratrol compared with mitogen alone (\(P < 0.01\)), and trends for dose-responsive increases in Bcl-2 expression and caspase-3 activation were observed (\(P\)-trend < 0.0001). Differences in IgM and IgG production were not observed for PBMC treated with resveratrol. Kaempferol at 10 \(\mu\)mol/L slightly inhibited proliferative responses (\(P < 0.05\)) but did not affect B cell function or apoptosis. Quercetin did not alter B cell proliferation, function, or apoptosis. These data show that human B lymphocyte proliferation and apoptosis are modified by physiological concentrations of resveratrol and suggest that exposure of human B cells to resveratrol may increase survival by upregulating Bcl-2.


Introduction

Resveratrol (3,5,4\textsuperscript{-trihydroxy-\textit{trans}}-stilbene) is a plant antibiologic that functions to protect the plant from fungal infection (1). Resveratrol is present in the skins, seeds, and leaves of grapes and a narrow range of other foods, including peanuts, blueberries, and cranberries. Quercetin and kaempferol are 2 related flavonols found in many fruits and vegetables (2). Resveratrol, quercetin, and kaempferol have shown antiinflammatory activities [reviewed in (3,4)]. Resveratrol, quercetin, and kaempferol can inhibit the activation of the transcription factor nuclear factor-\(\kappa\)B, an important mediator of inflammatory responses, as well as several enzymes involved in signal transduction pathways (1,5,6). Resveratrol and quercetin inhibit the enzyme cyclooxygenase-2, which is induced by mitogens, cytokines, and bacterial lipopolysaccharide and plays a key role in inflammatory processes (1,7).

The humoral immune response is mediated by activation of T lymphocytes through interaction with antigen-presenting cells. After activation, T lymphocytes promote the activation of B lymphocytes and production of Ig specific for the antigen presented. Production and secretion of Ig by activated B lymphocytes is regulated by antigen interaction with the B cell receptor, direct cell-to-cell contact with T lymphocytes, and production of cytokines by T helper cells (8). T helper type 1 lymphocytes produce the cytokines interleukin (IL)\textsuperscript{3-2} and interferon-\(\gamma\) that promote the generation of CD8\(^+\) cytotoxic T cells (9). T helper type 2 cells produce the cytokines IL-4, IL-5, and IL-10 that promote B cell proliferation and production of Ig (9,10).

Apoptotic cell death is mediated by the sequential activation of caspases, and apoptotic cells exhibit a variety of morphological changes characterized by chromatin condensation, DNA fragmentation, membrane blebbing, and formation of apoptotic bodies that are eventually phagocytosed in vivo (11). Activation of caspase-3 is an early to intermediate event during apoptosis that is necessary to induce DNA fragmentation and changes in cellular morphology. Caspase-3 mediates the cleavage of the majority of cytoplasmic and nuclear proteins, including those responsible for maintaining cytoskeletal structure (12). The

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B cell lymphoma-2 (Bcl-2) family of proteins contains both anti- and proapoptotic proteins. Bcl-2 and Bcl-XL are antiapoptotic members of the Bcl-2 family of proteins and are expressed in B lymphocytes (13,14). Each of these proteins is partly responsible for maintaining viability after B cell activation.

Sharma et al. (15) showed resveratrol inhibited proliferation of activated T and B cells isolated from mouse spleens and decreased production of Ig. However, significant differences were observed only at resveratrol concentrations of 20 μmol/L. To date, no research has been performed to define the effects of resveratrol, quercetin, or kaempferol on activated human B lymphocytes. We hypothesized that due to other antiinflammatory activities ascribed to these polyphenolic compounds, they would inhibit activation, survival, and function of human B lymphocytes. In this study, we collected peripheral blood mononuclear cells (PBMC) from healthy human volunteers and activated these cells with a polyclonal B cell mitogen to induce proliferation and Ig synthesis in the absence and presence of physiological concentrations of resveratrol, quercetin, and kaempferol. Proliferative responses, expression of Bcl-2, activation of caspase-3, and production of IgM and IgG were measured to determine whether there was suppression of B lymphocyte activation and survival by these polyphenols.

Materials and Methods

Participants and cell preparation. Twelve healthy adult human volunteers between the ages of 18 and 60 y (5 male, 7 female) were recruited to participate in the study. The human study protocol outlining the ethical treatment and protection of human subjects was approved by the Institutional Review Board at the University of California, Davis, CA. The protocol and goals of the study were explained to each volunteer and written consent was obtained. Potential volunteers were excluded if they were overtly ill, had disease states affecting immune cell function (allergy, asthma, autoimmune disorders, AIDS), or were taking doctor-prescribed medications that affected immune cell responsiveness. Blood was collected at ~1000 h into CPT tubes (Becton Dickinson) from each nonfasting volunteer by venipuncture and PBMC were isolated using the manufacturer's protocol. The plasma was discarded and the cells were washed once with PBS. Platelets were removed by centrifugation at 200 × g for 10 min before growth medium was added to the cells. Growth medium consisted of RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 60 mg/L penicillin, 100 mg/L streptomycin, 0.25 mg/L amphotericin B, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine (Invitrogen), and 50 μmol/L β-mercaptoethanol (Sigma).

Reagents and antibodies. Resveratrol and kaempferol aglycones and the PKH67GL kit were purchased from Sigma. Quercetin aglycone was isolated from Calbiochem. Resveratrol, quercetin, and kaempferol were dissolved in dimethylsulfoxide (DMSO; Sigma) and stored at -20°C. Pokeweed mitogen (PWM; Sigma) was dissolved in PBS and stored at a concentration of 1 g/L at -20°C. Fluorescein isothiocyanate (FITC)-conjugated anti-human activated caspase-3, PE-conjugated anti-human Bcl-2, and allophycocyanin (APC)-conjugated anti-human CD19 were purchased from Becton Dickinson. Control antibodies used included FITC-conjugated rabbit IgG (eBioscience), PE-conjugated hamster IgG1 (Becton Dickinson), and APC-conjugated mouse IgG1 (Becton Dickinson).

Proliferation assay. Proliferation responses were measured by staining PBMC with PKH67 following the manufacturer's protocol. The concentration of PWM that induced maximum proliferation of B lymphocytes was determined by testing different doses. For analysis of the effects of polyphenols on B cell growth, PBMC were plated in duplicate at 100,000 cells per well in 96-well, flat-bottomed microtiter plates in the presence or absence of PWM (final concentration of 5 μg/L).

Resveratrol, quercetin, or kaempferol were added to the appropriate wells at final concentrations of 2, 5, and 10 μmol/L. Control wells for proliferation contained PWM and 0.1% DMSO (equivalent to DMSO concentration used for resveratrol, quercetin, or kaempferol). Control wells for nonproliferating cells contained 0.5% PBS and 0.1% DMSO. The final volume per well was 200 μL. Cells were incubated for 6 d at 37°C, 5% CO2. Cells from duplicate wells were pooled and stained with APC-conjugated anti-CD19 antibody for 30 min on ice to identify the B lymphocyte population in each sample. Cells were washed once, fixed in 1% paraformaldehyde prepared in PBS, and collected on a FACSCanto fluorescence-activated cell sorter using FACSDiva software (Becton Dickinson). Fifty thousand events were collected for each sample. All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells. Proliferation was measured on CD19+ gated cells using the Proliferation Wizard feature of ModFit LT software (Verity Software House). For each treatment group, comparisons were made between the numbers of cells remaining in the unproliferated state (parent) and the numbers of cells in subsequent generations. Unstimulated CD19+ cells were used to locate the parent peak for calculation of generations in the PWM-stimulated cells.

Bcl-2 expression and caspase-3 activation. PBMC were plated in duplicate and incubated for 6 d at 37°C as described above before analysis of Bcl-2 and caspase-3. Cells from duplicate wells were pooled, washed once, and stained with APC-conjugated anti-CD19 for 30 min on ice. Cells were washed then fixed and permeabilized using Cytofix/ CytoPerm solution (Becton Dickinson) according to the manufacturer’s protocol. Cells were washed and stained with FITC-conjugated anti-caspase-3 and PE-conjugated anti-Bcl-2 antibodies for 30 min on ice. Cells were washed, refixed in 1% paraformaldehyde, and fluorescence was measured by flow cytometry. Fifty thousand events were collected for each sample. All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells. Bcl-2 expression and caspase-3 activation were measured in B lymphocytes by gating the CD19+ population. Isotype controls for the anti-Bcl-2 and anti-caspase-3 antibodies were used to determine marker settings in the fluorescence-activated cell sorter software.

IgM and IgG production. PBMC were plated in duplicate as described above and were incubated for 8 d at 37°C and supernatants were pooled from duplicate wells. Supernatants were centrifuged at 900 × g for 10 min to remove cells and debris and aliquots were stored at -20°C. ELISA kits for measuring human IgM and IgG were purchased from Bethyl Laboratories. ELISA were performed according to the manufacturer’s protocol. Measurements for each supernatant were performed in duplicate on a Synergy 2 microplate reader using Gen5 software (BioTek Instruments).

Statistical analysis. All statistical analyses were performed with GraphPad Software. One-way repeated-measures ANOVA with Dunnett’s post hoc test and linear trend post tests were used to compare proliferation, Ig production, Bcl-2 expression, and caspase-3 activation in response to different doses of resveratrol, quercetin, or kaempferol. Values in the text are means ± SEM. Differences were considered significant at P < 0.05.

Results

Differential proliferative response of B lymphocytes to resveratrol. PWM is a T cell-dependent mitogen that induces proliferation and Ig synthesis in B lymphocytes (16). Maximum proliferative response of B lymphocytes to PWM was reported to be 6 d (17). Unstimulated and PWM-stimulated human PBMC were cultured for 6 d in the presence or absence of 2, 5, or 10 μmol/L resveratrol, quercetin, or kaempferol. PBMC were stained with fluorochrome-conjugated anti-CD19 antibody to identify the B lymphocyte subpopulations. The mean percent of CD19+ B lymphocytes in the unstimulated PBMC population

1604 Zunino and Storms

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was 5.4 ± 0.3%. The proportion of CD19+ B lymphocytes increased to 15.2 ± 1.4% in PWM-stimulated PBMC treated with 5 μmol/L resveratrol compared with 11.5 ± 1.1% in cells treated with PWM alone (P < 0.01). PKH67 dye was used to label the cell membranes before treatments, and the subsequent loss of fluorescence was used to determine the percent of CD19+ cells in each generation compared with unstimulated cells, where a distinct parent population of B cells was present. A trend for decreases in the percent of CD19+ lymphocytes remaining in the parent population after PWM stimulation was observed with 2 and 5 μmol/L resveratrol treatments compared with cells stimulated with PWM alone (Fig. 1) (P-trend = 0.01). However, resveratrol at a final concentration of 10 μmol/L increased the percent of PWM-stimulated CD19+ B lymphocytes that remained in the parent population compared with PWM control cells (Fig. 1) (P < 0.01). Furthermore, treatment with 10 μmol/L resveratrol decreased the percent of CD19+ cells in the 7th and 8th generations compared with the PWM-stimulated control cells (P < 0.05). Kaempferol at a final concentration of 10 μmol/L slightly increased the percent of CD19+ cells in the second generation to 8.9 ± 1.1% compared with 6.5 ± 0.7% in cells stimulated with PWM alone (P < 0.05). Treatments with quercetin did not alter the proliferative responses of PWM-stimulated CD19+ cells compared with cells stimulated with PWM alone.

Expression of Bcl-2 and activation of caspase-3. Expression of the antiapoptotic protein Bcl-2 and the activation status of the proapoptotic protein caspase-3 were measured in CD19+ lymphocytes after 6 d of treatment in the presence or absence of PWM, resveratrol, quercetin, or kaempferol. The percent of CD19+ cells positive for Bcl-2 or activated caspase-3 and the fluorescent intensity of these intracellularly stained proteins were used to determine the relative levels of Bcl-2 and activated caspase-3 proteins. The percentage of cells expressing Bcl-2 increased in PWM-stimulated cells treated with 10 μmol/L resveratrol compared with PWM-treated control cells (Fig. 2A) (P < 0.01). The expression level of Bcl-2 protein as measured by median fluorescence intensity also increased in cells incubated with 10 μmol/L resveratrol compared with PWM-treated control cells (Fig. 2B) (P < 0.01). Trends for dose-dependent increases in both Bcl-2 expression and intensity of Bcl-2 fluorescence were observed in PWM-stimulated cells treated with 2, 5, and 10 μmol/L resveratrol (P-trend < 0.0001). The percent of cells with activated caspase-3 and the median fluorescence intensity of the caspase-3 signal increased in PWM-stimulated cells treated with 10 μmol/L resveratrol compared with PWM-treated control cells (Fig. 3A, B) (P < 0.01). A trend for dose-dependent increases in the percent of cells with activated caspase-3 and median fluorescence intensity was observed in CD19+ B cells treated with 2, 5, and 10 μmol/L resveratrol (P-trend < 0.0001). The percent of cells that were double positive for Bcl-2 and activated caspase-3 increased in PWM-stimulated cells treated with 10 μmol/L resveratrol compared with PWM-treated control cells (Fig. 4) (P < 0.01). A trend for dose-responsive increases in the percent of cells that were double positive for Bcl-2 and caspase-3 was observed for cells treated with 2, 5, and 10 μmol/L resveratrol (P-trend < 0.0001) (Fig. 4). Quercetin and kaempferol did not alter Bcl-2 expression or caspase-3 activation in PWM-stimulated CD19+ cells compared with cells treated with PWM alone.

**Discussion**

In this study, proliferation, apoptosis, and function of activated human B lymphocytes were analyzed in response to physiolog-
ical concentrations of the polyphenols resveratrol, quercetin, and kaempferol. To our knowledge, this is the first report describing the effects of these polyphenols on activated human B lymphocyte proliferation and function. We showed that resveratrol modulated B lymphocyte proliferation and apoptosis but did not affect Ig synthesis. Quercetin and kaempferol did not substantially affect B lymphocyte proliferative or apoptotic responses, nor did they affect Ig synthesis. Sharma et al. (15) described the activity of resveratrol at doses of 1, 5, 10, and 20 \( \mu \text{mol/L} \) on the in vitro responses of activated mouse B cells and the synthesis of Ig after stimulation with lipopolysaccharide. In these studies with mouse splenocyte cultures, the authors observed significant inhibition of T and B cell proliferation, reduced IL-4 and interferon-\( \gamma \) synthesis, increased IL-10 secretion, and decreased production of IgG1 and IgG2a at a dose of 20 \( \mu \text{mol/L} \) resveratrol. It should be noted that the authors claimed a dose-dependent response for the effect of resveratrol on cytokine synthesis and proliferative response. However, no trend analyses were performed to substantiate these observations. In our study, a differential proliferative response in the activated human CD19+ B cell population was observed with 2 and 5 \( \mu \text{mol/L} \) compared with 10 \( \mu \text{mol/L} \) resveratrol. At a concentration of 5 \( \mu \text{mol/L} \), resveratrol increased the overall number of B lymphocytes after 6 d of stimulation with PWM. Furthermore, there was a trend toward a reduction of B cells in the parent population in the presence of 2 and 5 \( \mu \text{mol/L} \) resveratrol, suggesting an increase in proliferative response with the lower resveratrol concentrations. However, 10 \( \mu \text{mol/L} \) resveratrol clearly decreased proliferation of B lymphocytes as indicated by the increased number of cells remaining in the parent population and reduction of cells in the 7th and 8th generations compared with PWM-stimulated control cells.

Treatment of activated human B cells with resveratrol increased the expression of Bcl-2, an important antiapoptotic protein expressed in activated B and T cells. The fluorescence intensity of anti-Bcl-2 antibody was used to indicate relative amounts of Bcl-2 molecules per cell compared with control cells. Both the fluorescence intensity and the percentage of CD19+ cells with Bcl-2 increased after resveratrol treatment. It was shown that B and T cells that overexpressed Bcl-2 had delayed cell cycle entry that resulted in increased survival (18,19).

Delayed cell cycle entry induced by Bcl-2 was associated with increased expression of the cell cycle inhibitor p27Kip1 as well as the retinoblastoma family member p130 (20,21). Furthermore, inhibition of cell cycle entry was linked to tyrosine residue 28 in the BH4 domain of Bcl-2 that is independent of the protein’s antiapoptotic functions (22). Human CD19+ B cells treated with 10 \( \mu \text{mol/L} \) resveratrol had a significant increase in the expression levels of Bcl-2 and a larger percentage of cells remained in the nonproliferating parent population compared with the PWM-stimulated control cells. We also observed a dose-responsive trend toward increased Bcl-2 expression. It is unclear why this trend existed considering the apparent increase in proliferative response in cells treated with 2 and 5 \( \mu \text{mol/L} \) resveratrol. However, B cells treated with 5 \( \mu \text{mol/L} \) resveratrol did have an overall increase in the number of B cells compared with the other treatment groups, which may argue for increased survival after activation when cells are exposed to lower resveratrol concentrations. An increase in activation of caspase-3 was also observed in B cells treated with 10 \( \mu \text{mol/L} \) resveratrol and the increased caspase-3 activation was observed in Figure 4.
in B lymphocytes with upregulated Bcl-2. Activated B cells are normally recruited to the germinal centers where hypermutation of the Ig gene creates B cell populations with increased specificity for antigen (23). B cells producing low affinity antibodies in the germinal center, as well as those in a cell culture system, undergo apoptosis and this apoptotic death can be blocked by Bcl-2 (24).

Taken together, these data suggest that resveratrol may promote survival of activated B lymphocytes by upregulating expression of Bcl-2, which can act as an inhibitor of apoptosis as well as a cell cycle regulator.

Studies have been performed in humans and animals to determine the tissue distribution, excretion rates, and general bioavailability of resveratrol after oral or i.g. administration. In humans, after 25 mg of 14C-labeled resveratrol was given as a single oral dose, the concentration of resveratrol peaked at ~500 µg/L in the plasma (2 µmol/L) at 1 h, with a second peak of ~300 µg/L (1.3 µmol/L) at 6 h (25). In these experiments, there was at least 70% absorption of resveratrol and most of the oral dose was recovered in the urine. Although the plasma levels were low with the single oral dose of 25 mg, absorption was rapid and efficient and the plasma half-life of resveratrol and metabolites was calculated at 9.2 h. The major forms of resveratrol observed in this study were sulfate and glucuronide conjugates. In a phase I human trial, single oral doses ranging from 0.5 to 5 g resveratrol achieved plasma concentrations of the aglycone parent compound from 0.3 to 2.4 µmol/L (26). Plasma concentrations of glucurononitated and sulfated resveratrol metabolites ranged from ~1 to 4 µmol/L and 4 to 14 µmol/L, respectively. In rat studies, an oral dose of 50 mg resveratrol/kg body weight achieved a concentration of ~10 µmol/L resveratrol aglycone in the plasma that became undetectable by 12 h (27).

However, the glucurononitated resveratrol metabolite reached a plasma concentration of ~100 µmol/L. 14C-labeled resveratrol at a single oral dose of 5 mg/kg body weight showed distribution in the duodenum, colon, liver, kidney, lung, spleen, heart, brain, and testis of mice by 2 h, but the aglycone (parent) form of resveratrol was the main product isolated from the tissues. Longer term experiments utilizing rats showed the presence of resveratrol metabolites in the plasma after an 8-wk feeding of 300 mg resveratrol/kg body weight-d (30). The concentrations of the different resveratrol conjugates in the plasma ranged from 0.37 to 7.46 mg/L (~1–25 µmol/L) by the end of the 8-wk feeding study. Taken together, these data suggest that B lymphocytes in the blood, and possibly hematopoietic tissues, may be exposed to physiologically achievable concentrations of resveratrol used in our study.

Resveratrol appears to protect activated human B lymphocytes from apoptosis by upregulating the antiapoptotic protein Bcl-2. These results are in contrast to the myriad of publications showing resveratrol treatment downregulates Bcl-2 (1). However, much of this work has been performed in vitro using cancer cell lines or in vivo using animal models for cancer. Resveratrol modified the proliferative response and status of proteins involved in apoptosis in normal human B lymphocytes. Therefore, resveratrol, either obtained from dietary sources or as a supplement, may have a considerable influence in the survival of activated B cells during a humoral response to foreign antigen.

**Literature Cited**


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