Effects of Resveratrol on Lymphocyte Proliferation and Cytokine Release

Paolo Boscolo,1 Antonella del Signore,2 Enrico Sabbioni,3 Mario Di Gioacchino,1 Luca Di Giampaolo,1 Marcella Reale,4 Pio Conti,4 Roberto Paganelli,1 and Mario Giaccio 2

1Department of Medicine and Science of Aging, 2 Department of Sciences, and 3 Department of Oncology and Neurosciences, University G. D’Annunzio, Chieti and Pescara, Italy; 4 European Commission, Institute for Health and Consumer Protection, ECVAM Unit, Ispra, Italy.

Abstract. Resveratrol, synthesized in dietary plants and contained in wine, has been reported to play a beneficial role in certain cardiovascular regulatory mechanisms and to inhibit carcinogenesis by activating immune and inflammatory responses and apoptosis. The object of this study was to elucidate the “in vitro” effects of different concentrations of resveratrol (10⁻⁴, 10⁻⁵, and 10⁻⁷ M) on human peripheral blood mononuclear cell (PBMC) proliferation and cytokine release. Spontaneous PBMC proliferation was unaffected by resveratrol, while the compound at 10⁻⁴ M inhibited (69 %) the PHA-stimulated PBMC proliferation. The proliferation stimulation index (ie, the ratio of PHA-stimulated PBMC proliferation/spontaneous PBMC proliferation) of cultures containing 10⁻⁴ M resveratrol was very low in relation to the control, while the proliferation stimulation index values at 10⁻⁵ and 10⁻⁷ M were similar and slightly higher (without statistical significance), respectively. At 10⁻⁴ M, resveratrol strongly inhibited PHA-stimulated IFN-γ and TNF-α release from PBMC, but it did not cause inhibition at 10⁻⁵ or 10⁻⁷ M. The concomitant immune effects of resveratrol on PBMC proliferation and release of IFN-γ and TNF-α may be explained by an inhibitory effect on transcription factor NF-kappaB. This study suggests that resveratrol, which is typically present in red wine at about 10⁻⁵ M, is unlikely to cause inhibitory immune effects. However, a stimulatory effect of low concentrations of resveratrol on the immune system cannot be excluded. (received 1 February 2003; accepted 6 February 2003)

Keywords: resveratrol, lymphocytes, macrophages, IFN-γ, TNF-α, immunotoxicity

Introduction

It has been demonstrated that polyphenols (and in particular 3,5,4′-trihydroxystilbene, resveratrol) synthesized in dietary plants exert several biological activities [1,2]. Resveratrol is produced by some spermatophytes and is found in grapes, wines, Polygonum roots, peanuts, and berries [1-4]. Four isomeric forms of resveratrol have been identified: trans- and cis-resveratrol and trans- and cis-β-D-glucopyranoside; these isomeric forms constitute the “total stilbene.” Trans-resveratrol and trans- and cis-β-D-glucopyranoside are present in grapes, whereas all 4 forms are contained in wines. Trans-resveratrol was first detected in grapevines (Vitis vinifera) in 1976 by Langcake and Pryce [3], and its presence was explained as a response to fungal infection, mainly Botrytis cinerea. In 1992 Siemann and Creasy [4] suggested that this compound might be a biologically active constituent of red wine, possibly beneficial for human health. Since then, the content of resveratrol in wines and its properties have been extensively investigated. Recent articles report high variability in resveratrol concentrations depending on wine processing [5], climatic factors that affect
grape cultivation [6], yeasts used in the fermentative processes [7], and various other factors [8].

Kuhnle et al [9] demonstrated that resveratrol, which passes through enterocytes of the jejunum and ileum, is not metabolised. During passage through enterocytes, 96.5% of absorbed resveratrol is conjugated with glucuronic acid. Glycosidase activity that converts glucosides to aglycones is exerted during the digestive phase. It was suggested that all of the isoforms (constituting the “total stilbene”) are transformed to resveratrol in the digestive tract by this activity; therefore, resveratrol may act “in vivo” as the only biologically active form.

The resveratrol content of red wine (approximately 10^-5 M [6]) has been reported to afford cardiovascular protection and to reduce atherosclerosis by various mechanisms [10,11]. These include modulation of lipid turnover, production of eicosanoids, oxidation of lipoproteins, and reduction of platelet adhesion [12,13]. Moreover, resveratrol was reported to inhibit angiotensin-II-induced hypertrophy of vascular smooth cells [14]. Resveratrol has also been reported to be involved in anti-inflammatory and anticancer mechanisms [15]. It apparently affects different stages of carcinogenesis in murine mammary epithelial cells [16]; it limits the ability of human colon carcinoma cells to form colonies through both pro-apoptotic and non-apoptotic mechanisms [17].

Resveratrol has also been reported to reduce the risk of cardiovascular and tumoral diseases by acting on the mechanisms that regulate the expression of growth factors and cytokines such as transcription factor NF-kappaB [2,16]. Studies on immune effects of resveratrol have been performed. The compound was shown to inhibit the proliferation and IL-2 and interferon (IFN)-γ production of splenic lymphocytes, the activity of cytotoxic T lymphocytes, the activity of lymphokine-activated killer cells, as well as the production of TNF-α and IL-12 by peritoneal macrophages [18] . Resveratrol was also reported to exert “in vitro” biphasic immune actions, i.e., inhibition at high concentration of cytotoxic T lymphocyte and NK cell activities and the production of IFN-γ, IL-2, and IL-4 by T lymphocytes, and stimulatory immune actions at low concentrations [19].

The object of this study was to elucidate the “in vitro” immune effects of different concentrations of resveratrol on peripheral blood mononuclear cell (PBMC) proliferation and cytokine release.

Materials and Methods

Resveratrol. Resveratrol solution (10^-3 M) was prepared by diluting 22.82 mg of trans-resveratrol (Sigma Chemical Co, Milan, Italy) in 100 ml of distilled water containing 10% (v/v) of ethanol. This solution was diluted to 10^-4, 10^-5, and 10^-7 M in cultures containing ethanol concentrations from 1 to 0.001%.

Isolation of human PBMC. Nine healthy men (mean age 34 yr, range 24-58 yr) were recruited for the study. They were unaffected by disease and did not use any drugs. Fasting venous blood samples were obtained from each subject at 8 am. Human PBMC were purified from EDTA-treated whole blood by Ficoll-Hypaque (BioSpa, Milan, Italy) density gradient centrifugation (20 min at 400 x g). After three washings, PBMC were resuspended in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma). This mixture is designated as “complete medium.”

Cell proliferation. PBMC were suspended at 10^6 cells/ml in complete medium. Aliquots (100 µl) of cell suspension were placed in each well of a standard 96-well microtiter plate (Falcon, St. Louis, MO). The cells were incubated for 78 hr at 37°C in a humidified atmosphere with 5% CO_2 under the following conditions:
(a) without addition of phytohemagglutinin (PHA) and resveratrol (control sample),
(b) stimulation by 20 µg/ml PHA without resveratrol (control sample),
(c) as reported in (a) and (b) with the addition of 0.1 ml of ethanol (1%, v/v), and
(d) containing 10^-4, 10^-5, or 10^-7 M resveratrol with and without PHA stimulation.

Quantification of cell proliferation. Proliferation was evaluated using the BrdU cell proliferation assay.
During the final 24 hr of culture, BrdU was added to wells of the microtiter plate. Cells were fixed and permeabilized and DNA was denaturated by treatment for 30 min at room temperature with fixative/denaturing solution. Anti-BrdU monoclonal antibody was pipetted into the wells and allowed to incubate for 1 hr. Unbound antibody was washed away and horseradish peroxidase-conjugated goat anti-mouse antibody was added for 30 min at room temperature. Contents of the wells were removed by inverting over a sink and tapping on paper towels. Chromogenic substrate solution, tetramethyl benzidine (TMB), was added to each well and incubated in the dark at room temperature for 15 min. Stop solution was added to each well in the same sequence as the previous addition of substrate solution. All of the reagents were provided with the kit and used in accordance with the manufacturer's instructions. Experiments were performed in triplicate. The absorbance of the contents of each well was measured using a spectrophotometric microtiter plate reader at dual wavelengths (450 and 540 nm). The color intensity was proportional to the amount of incorporated BrdU in the cells and thus to the degree of cell proliferation.

Production and measurement of cytokines. Cultures were set up in 24-well Costar plastic microtiter plates (well volume, 1 ml), using 0.8 ml of PBMC (containing 10⁶ cells) in complete medium under the following conditions:

- (a) without addition of PHA or resveratrol (control),
- (b) stimulation by 10 µg/ml PHA without resveratrol (control),
- (c) as reported in (a) and (b) with addition of 0.1 ml of ethanol (10% v/v), as controls for the cultures containing 10⁻⁴ M resveratrol and 1% of ethanol,
- (d) stimulation by 10 µg/ml PHA in presence of 10⁻⁴, 10⁻⁵, or 10⁻⁷ M resveratrol.

The cultures were incubated at 37°C in humidified atmosphere with 5% CO₂ for 48 hr; cells were checked for viability by trypan blue dye exclusion using an inverted Leica microscope. Supernatants were collected and stored at -70°C in aliquots until analysis. Interferon (IFN)-γ, and tumor necrosis factor (TNF)-α levels in the culture supernatants were determined by Quantikine ELISA kits (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions.

Statistical analysis. Statistical analyses were performed with Statistica software (release 4.5). The Kolmogorov-Smirnov test was used to evaluate the data distributions.

Results

The Kolmogorov-Smirnov test showed that most of the experimental data had non-parametric distributions. In particular, values for cytokine release conformed more to a parametric distribution when they were expressed as a percent of the corresponding controls, rather than as units (pg/ml).

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<th>Spontaneous PBMC proliferation</th>
<th>PHA-stimulated PBMC proliferation</th>
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<tr>
<td></td>
<td>Absorbance (units)</td>
<td>Ratio (%) with/without resveratrol</td>
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<tr>
<td>Without resveratrol</td>
<td>170 ± 29</td>
<td>1,633 ± 214</td>
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<tr>
<td>With 10⁻⁴ M resveratrol</td>
<td>148 ± 21</td>
<td>89.1% ± 11.4</td>
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<tr>
<td>With 10⁻⁵ M resveratrol</td>
<td>171 ± 32</td>
<td>104.7% ± 17.1</td>
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<tr>
<td>With 10⁻⁷ M resveratrol</td>
<td>156 ± 18</td>
<td>93.6% ± 8.9</td>
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</table>

* Significant difference vs control (p < 0.001) by Mann-Whitney U test
Spontaneous and PHA-stimulated PBMC proliferation, as well as IFN-γ and TNF-α release from PBMC, were not influenced by the 1% ethanol content of the cultures.

Table 1 and Fig. 1 show data for spontaneous and PHA-stimulated PBMC proliferation; Fig. 2 shows data for PHA-stimulated release of IFN-γ and TNF-α from PBMC. The following observations were made:

The spontaneous PBMC proliferation assay without resveratrol (baseline profile) shows an absorbance (170 ± 29 units) that increases to 1633 ± 214 units in PHA-stimulated cultures (mean ± SD; Table 1). Thus, an increase of 9-10 fold is the control response to PHA.

Spontaneous PBMC proliferation is not modified at concentrations of 10⁻⁴, 10⁻⁵, or 10⁻⁷ M resveratrol (Table 1). Addition of 10⁻⁴ M resveratrol leads to inhibition of 69.4% of the proliferation of the PHA-stimulated PBMC, while exposure of PBMC to 10⁻⁵ or 10⁻⁷ M resveratrol does not affect the PHA-induced proliferation (Table 1).

The stimulation index (SI) of blastogenesis (ratio of PHA-stimulated PBMC proliferation/spontaneous PBMC proliferation) of control cultures is 9.46 ± 1.25, while for those containing 10⁻⁴ M resveratrol the SI is 2.69 ± 0.81 (p <0.001) (Fig. 1); there is no effect at 10⁻⁵ M resveratrol. On the other hand, the SI observed at 10⁻⁷ M resveratrol is 10.72 ± 0.99, which is slightly higher (but without statistical significance) in relation to the control (Fig. 1).

Spontaneous IFN-γ release from PBMC was close to the detection limit (7 ± 6 pg/ml), while the PHA-stimulated release was 1205 ± 305 pg/ml; spontaneous TNF-α release from PBMC was 104 ± 63 pg/ml and the PHA-stimulated release was 2206 ± 551 pg/ml.

At 10⁻⁴ M, resveratrol almost completely inhibited PHA-stimulated release of IFN-γ from PBMC (12.1 ± 10.4% of the control) (Fig. 2) and that of TNF-α (21.6 ± 14.9% of the control), while the exposure to 10⁻⁵ and 10⁻⁷ M resveratrol did not exert effects (Fig. 2).

Discussion

This study shows that 10⁻⁴ M resveratrol strongly inhibits PHA-stimulated PBMC proliferation and cytokine release, while it does not exert these effects at 10⁻⁵ M. The content of trans-resveratrol in wines is about 1-2 mg/L (about 0.5 x 10⁻⁵ M), whereas the content of “total stilbene” (trans- and cis-resveratrol and trans- and cis-β-D-glucopyranoside) is typically in the range of 5-10 mg/L (about 2-5 x 10⁻⁵ M) [6]. It has been suggested that “total stilbene” of red wine is transformed to resveratrol in the intestinal tract before it is absorbed as glucuronide [9]. Therefore, it seems unlikely that low intake of wine would exert immune effects, while
high intake of wine (>1 L/day) may inhibit immune functions related to PBMC proliferation and cytokine production.

PHA-stimulated PBMC proliferation needs the activation of several metabolic functions, including IL-2 production and release [20]. It is known that TNF-α is produced by a variety of cell types including T and B lymphocytes, NK cells, macrophages, astrocytes, and dendritic cells [21]. This cytokine exerts several important biological activities including those involved in septic shock and chronic diseases such as rheumatoid arthritis; it is also shown to be cytotoxic for many tumor cells, inducing apoptosis [22]. On the other hand, IFN-γ production (mainly due to T lymphocytes) may be considered the expression of a Th-1 type cell response [23]. In this regard, the immune effects of resveratrol, concomitantly acting on PBMC proliferation and production of IFN-γ and TNF-α, may be explained by its inhibitory effect on the transcription factor NF-kappaB [2,16]. This transcription factor is strongly linked to immune and inflammatory responses, regulation of cell proliferation, and apoptosis. For this reason, involvement of resveratrol in anti-cancer mechanisms may be considered; possible use of resveratrol as a chemotherapeutic agent may merit investigation in accordance with the results and suggestions of other authors [15-17].

Resveratrol may interact with metals and chemical compounds present in wine or constituents of the diet. In this regard, it was found that ethanol, present in wine at concentrations from 8 to 14%, potentiated the immune effects of resveratrol on murine macrophages [24].

In conclusion, this study demonstrates that resveratrol inhibits immune functions only at high concentrations. Moreover, this compound does not exert effects at low concentrations that might be likely to reduce the risks of degenerative [10-14] and neoplastic [15-17] diseases. On the other hand, it cannot be excluded that resveratrol, at low concentrations, might act on transcription factors (e.g., NF-kappaB), and concomitantly affect immune, inflammatory, and apoptotic mechanisms [2-16].

References