Resveratrol diminishes platelet aggregation and increases susceptibility of K562 tumor cells to natural killer cells

Ioannis K Toliopoulos1,2, Yannis V Simos1,2, Stergios Oikonomidis1,2 and Spyros C Karkabounas1
1Laboratory of Physiology, Faculty of Medicine, University of Ioannina, Ioannina 45110, Greece
2Konstantinion Research Center of Molecular Medicine and Biotechnology, Non-Profit Foundation, Thessaloniki, Greece

Received 31 May 2012; revised 19 December 2012

Platelet aggregation around migrating cancer cells protects them against the activity of natural killer cells (NKCs). The inability of immune system to respond results in the progression of malignant diseases. This study was designed to evaluate the effects of resveratrol (3, 4', 5-trihydroxystilbene) on platelet aggregation and NKCs activity. Experiments were designed to evaluate the platelet aggregation, production of thromboxane B2 (TXB2), estimation of expression of the platelet receptor GpIIb/IIIa (major biological markers for platelet aggregation) and functional activity of the NKCs against the K562 cancer cell line after incubation with various concentrations of reveratrol. Resveratrol at a concentration of 3 × 10⁻³M completely inhibited platelet aggregation (p<0.05), decreased TXB2 levels (p<0.05) and inhibited the expression of receptor GpIIb/IIIa in non-stimulated platelets (p<0.05). At the same concentration, it increased the NKCs cytotoxic activity at an average rate of 319 ± 34, 450 ± 34 and 62 ± 2.4% (p<0.05) in the NKC/targets cells ratios of 12.5:1, 25:1 and 50:1, respectively. Thus, resveratrol not only completely inhibited platelet aggregation and reduced TXB2 levels and expression of receptor GpIIb/IIIa, but also increased the cytotoxic activity of NKCs in vitro and thus increased the susceptibility of tumor cells to NKCs. Thus, resveratrol can be used as an additional supplement to modulate the immune system and to inhibit platelet aggregation in thromboembolic episodes. Further clinical investigation in vivo could lead to specific concentrations that may maximize the beneficial effect of resveratrol.

Keywords: Natural killer cells, Resveratrol, Platelets, Aggregation

Natural killer cells (NKCs) constitute the first line of defence against various infections and malignancies by rapidly recognizing and lysing a variety of malignantly transformed or virus-infected cells, without the need for either prior sensitization or major histocompatibility complex (MHC)-dependent recognition. NKCs participate in the clearance of viral infection, especially in the innate immune response that occurs in the early phase of infection. Their actions include destruction of infected cells, secretion of inflammatory cytokines and interaction with dendritic cells. NKCs also destroy malignant and virus-infected cells by direct contact and thus the migration of platelets around tumour cell protects them from NKCs lysis. Platelet aggregation is a process mediated by binding of fibrinogen to a glycoprotein called GpIIb/IIIa. When this binding is blocked by any inhibitors, then there is no platelet accumulation. Platelets also secrete organic substances, such as TXA2, which is later metabolized to TXB2 during the inflammation process and microparticles which regulate the action of other blood cells like lymphocytes.

Since most immunomodulatory chemical drugs are not suitable for chronic or preventive use, there is an increasing interest in identifying new immunomodulators that enhance non-specific host defence mechanisms. Polyphenols have been shown to possess various beneficial health properties. Research over the last 2 decades has shown the potential of resveratrol (3, 4', 5-trihydroxystilbene), a plant-derived polyphenolic compound as a novel class of non-toxic chemotherapeutic. However, some basic aspects of resveratrol action need to be clarified, before it can be developed into a clinically viable anticancer drug.
Earlier, a few studies have investigated the effect of resveratrol on NK activity. In this study, we have further evaluated the ability of resveratrol in increasing the susceptibility of tumor cells to NKCs through the modulation of platelet aggregation and NK cytotoxic activity in a series of in vitro and ex vivo experiments.

**Materials and Methods**

**Equipment**

Platelet aggregation was performed in the Ca-500 aggregometer (Chronolog Co., USA). Radioactivity of each sample was measured by using a γ-counter (Nucleus Co. Model 1600). The flow cytometer (Epics XL-MCL of Beckman-Coulter, USA) was used for NKCs measurements.

**Chemical and reagents**

Epinephrine (EPN), adenosine phosphate (ADP), arachidonic acid (ARA), thrombin (THR), resveratrol (R), phosphocreatine, creatine phosphokinase, acetylsalicylic acid and gingolides A and B, Ficoll, phosphate-buffered saline and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO, USA).

TXB$_2$ levels were estimated by the TXB$_2$/2,3-DINOR-TXB$_2$ radioimmune assay kit (Isotop Company, Institute of Isotops Co. Ltd. Budapest, Hungary). Expression of GpIIb/IIIa receptors of was measured by using the «ADI Aflo Platelet GpIIb/IIIa Occupancy» kit (American Diagnostics, Inc. USA). The evaluation of NKC cytotoxicity was done using the kit NKTEST$^{®}$ (ORPEGEN Pharma, Germany).

**Volunteers**

Twenty-eight healthy (non-smoking 18 male and 10 female volunteers with average age 45 yrs) accepted to donate blood for the completion of the experimental protocols of this study. The participation of volunteers in the study was without monetary compensation and in agreement with the human rights, legislation from the Declaration of Helsinki (1979) and approval of the Ethical Committee of University of Ioannina. The volunteers were free of medical problems in their history.

**Platelet rich plasma (PRP) preparation**

Whole blood (20 ml) was taken in the morning by free flow and transferred in plastic tubes that contained 3% citric nitrate. Blood samples were then centrifuged at 900 rpm for 10 min and PRP was isolated as supernatant. The rest of the blood samples were then centrifuged again at 3100 rpm for 15 min for the calibration of the aggregometer and platelet poor plasma (PPP) was collected as supernatant. The concentration of platelets was fixed at $2.5 \times 10^9$ cells/ml (Brecher-Cronkite’s method).  

**Platelet aggregation**

Platelet aggregation was induced by five different platelet stimulators (EPN, ADP, ARA, PAF and THR). The concentrations of platelet stimulators used to cause maximum non-reversible platelet aggregation were: EPN (5 µM), ADP (12 µM), ARA (0.7 µM), PAF (15 µM) and THR (1 IU/ml). Five microliter from each agonist was added in 450 µl of PRP for each measurement. Same measurements were repeated after incubation of each sample of 450 µl PRP with 5 µl addition of resveratrol (3 × 10$^{-3}$ M to 10$^{-5}$ M) before administration of platelet stimulators. Platelet aggregation was estimated after 5 min as the percentage of the maximum non-reversible aggregation caused by the platelet stimulators.

**Estimation of platelet TXB$_2$ production**

TXB$_2$ is a major prostaglandin derivative product, which induces arterial contraction and platelet aggregation, thus the measurement of its levels was considered significant for this study. TXB$_2$ was estimated in PRP before platelet aggregation and 5 min after its initiation, with and without administration of resveratrol (10$^{-3}$ M to 3 × 10$^{-5}$ M), 1.25 mg of indomethacin was used to stop TXB$_2$ production by the arachidonic acid pathway enzymes. The samples were centrifuged at 5000 rpm/min for 5 min and supernatant was collected and processed as described previously.

**Estimation of platelet GpIIb/IIIa receptors expression**

Glycoprotein GpIIb/IIIa is the most potent inhibitor of platelet aggregation. Therefore, its estimation was an important biomarker for the evaluation of the inhibition of resveratrol in platelet aggregation in this study. Expression of GpIIb/IIIa receptor per platelet was estimated by flow cytometric analysis. The receptors per platelet were measured in the isolated PRP samples without addition of any platelet stimulator and in PRP samples, which were incubated for 5 min with resveratrol (10$^{-5}$ M to 3 × 10$^{-3}$ M).

**Isolation of peripheral blood mononuclear cells (PBMC)**

20 ml whole blood was collected in the morning from 12 volunteers (8 males and 4 females with
average age of 52 yrs) and transferred into tubes that contained heparin as anticoagulant. Isolation of PBMC was performed as described previously\textsuperscript{21}. The isolated cells were then diluted in complete medium solution and their number was set at 5 × 10\textsuperscript{6} cells/ml by using the hematocytometer. The suspension contained the population of NKCs remained at room temperature till use.

**NKC functionality**

In order to evaluate NKC functionality, chronic myeloid leukemia cells from K562 cell line were used as target cells (TCs). The TCs concentration was set at 10\textsuperscript{5} cells/ml and their membranes were labeled with green fluorescence by fluorescein isothiocyanate. Suspensions of NKCs and TCs were then mixed in NKC/TC ratios of 12.5: 1, 25: 1 and 50: 1 in a final volume of 200 µl. The samples were incubated for 2.5 h in a CO\textsubscript{2} incubator. Cells’ nuclei were labeled with red fluorescence by propidium iodide, in order to detect the apoptotic and necrotic TC. Estimation of cytotoxicity of NKCs was performed by flow cytometric analysis\textsuperscript{22,23}. Same measurements were repeated in presence of 50 µl of resveratrol (10\textsuperscript{-5} M to 3 × 10\textsuperscript{-3} M) in the suspension of NKC/TC in the above-mentioned ratios before the incubation stage.

**Statistical analysis**

Data distribution analysis was performed with the Shapiro-Wilk test, frequency distribution histograms and normal probability plots (PP and QQ plots). The analysis showed that there was a normal distribution between data, thus the statistical significance between data means was determined by Student’s t-test. P-values <0.05 were considered as significant (SPSS version 17.0, Chicago, USA).

**Results and Discussion**

Resveratrol showed a dose-dependent inhibition of platelet aggregation (Table 1) and inhibited platelet aggregation (induced by all platelet stimulators) more than 50% at the concentration of 10\textsuperscript{-3} M and 100% at the concentration of 3 × 10\textsuperscript{-3} M. Administration of 3 × 10\textsuperscript{-3} M resveratrol significantly decreased levels of TXB\textsubscript{2} secreted by stimulated platelets (p<0.05) (Fig. 1). Furthermore, expression of the receptor GpIb/IIa in non-stimulated platelets (88138 ± 4562 receptors per platelet) and in platelets treated with 3 × 10\textsuperscript{-3} M resveratrol (2518 ± 112 receptors per platelet) was significantly reduced (p<0.05). No statistical difference observed in concentrations 10\textsuperscript{-5} M, 10\textsuperscript{-4} M and 10\textsuperscript{-3} M (results not shown).

**Table 1—Inhibition (%) of platelet aggregation by various concentrations of resveratrol**

<table>
<thead>
<tr>
<th>Resveratrol (M)</th>
<th>EPN</th>
<th>PAF</th>
<th>ADP</th>
<th>ARA</th>
<th>THR</th>
</tr>
</thead>
<tbody>
<tr>
<td>10\textsuperscript{-5}</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10\textsuperscript{-4}</td>
<td>7 ± 2</td>
<td>13 ± 2</td>
<td>10 ± 4</td>
<td>5 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>10\textsuperscript{-3}</td>
<td>53 ± 8</td>
<td>76 ± 3</td>
<td>69 ± 3</td>
<td>53 ± 3</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>3 × 10\textsuperscript{-3}</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

[EPN, ADP, ARA, PAF and THR were used as platelet stimulators in concentrations that caused maximum non-reversible platelet aggregation. Platelet aggregation was estimated after 5 min of incubation with various doses of resveratrol. Data are presented as mean ± SD] ADP, adenosine phosphate; ARA, arachidonic acid; EPN, epinephrine; PAF, platelet activating factor; THR, thrombin. *Statistical significant different from pre-administration values (p<0.05)]

Cytotoxicity of NKCs significantly increased (p<0.05) in the presence of 3 × 10\textsuperscript{-3} M resveratrol in all three ratios (12.5:1, 25:1 and 50:1) (Table 2). In all the other tested concentrations of resveratrol, cytotoxicity remained stable and statistically not significant. When the suspension of target cells (TCs) was incubated with resveratrol only (without NKCs), cytotoxicity was estimated in average rate of 5.5%,
showing that resveratrol itself could not kill TCs (results not shown because not statistically significant).

Our results showed that resveratrol completely inhibited platelet aggregation at the concentration of 3 × 10^{-3} M and modulated the functionality of the NKCs in vitro.

TXB$_2$ is the final stable metabolite of the unstable TXA$_2$, which is produced by the platelet enzymic COX system via biochemical reactions, where oxygen free radicals production takes place. Our experiments showed that TXB$_2$ production was higher when platelets were stimulated by THR, ARA and PAF than EPN and ADP before platelet aggregation. Addition of resveratrol (3 × 10^{-3} M) significantly decreased TXB$_2$ production in all the samples stimulated by the five antagonists. It was also observed that with the increase in platelet aggregation, TXB$_2$ levels also increased after the administration of resveratrol.

Inhibition of TXB$_2$ production suggested that resveratrol might act as an anti-inflammatory factor inhibiting COX action. During an inflammatory process, free radicals are produced and resveratrol possibly inhibits the COX activity by scavenging oxygen free radicals. This resulted in decrease of TXA$_2$ production, inhibition of platelet activation and GpIIb/IIIa membrane receptor’s expression like other potent antioxidants. Furthermore, the number of GpIIb/IIIa receptors was significantly decreased after the addition of resveratrol (3 × 10^{-3} M) in non stimulated platelets. The reduction of expression of GpIIb/IIIa receptor prevented platelet aggregation in vitro. Moreover, this glycoprotein is considered a major biological marker in vascular function issues. Thus, there is great interest in the clinical development of agents that can bind to platelet GpIIb/IIIa, block fibrinogen binding and can be used in the prevention and management of thromboembolic disease states. As platelets are regulators of immune cells’ action during the immune response, possibly resveratrol might influence mechanisms of immune cells’ action through platelets and thus modulating the immune responses.

Addition of resveratrol (3 × 10^{-3} M) modified the NKCs in a manner that caused the increase of functionality against K562 TCs. One of the most important findings from this study were immunomodulating action of resveratrol observed in the ratios 12.5:1 and 25:1, where only a small number of NKCs was engaged against TCs. One possible explanation was that resveratrol initiated the activating receptors of NKCs and increased the motility of these cells. These receptors originate from the largest family called killer-cell immunoglobulin-like receptors (KIRs) and influence resistance to viral infections, non-viral pathogens, susceptibility to autoimmune diseases, complications of pregnancy, as well as outcome of haemopoietic stem-cell transplantation. Moreover, KIRs work together with the lectin-like receptors CD94-NKG2C (an activating receptor).

It has been shown that resveratrol act via NKG2D-dependent c-June-N-terminal kinase (JNK) and extracellular-regulated kinase (ERK-1/2 pathways). In this manner, NKCs become more active and activate their killing mechanisms directly against TCs. Resveratrol also exerts partial agonist activity to akydron hydrocarbon receptor (AhR) which is a prominent regulator of cellular toxic events, apoptosis, tumor promotion, cell cycle and many other cellular functions. The key finding of the present study was the in vitro determination of a specific concentration of resveratrol (3 × 10^{-3} M) exerting the multi-target action—inhibition of platelet aggregation and stimulation of NKCs. The importance of this finding was that a specific concentration of resveratrol could diminish platelet aggregation around cancer cells and also enhance the activity of NKCs, leading to an effective response of the immune system. Earlier, it is shown that immunomodulation of resveratrol alone or with IL-12 enhances perforin expression and cytotoxic activity by two pathways, while another study has shown how a hydrocarbon receptor (AhR) can act on NKCs and improve their cytotoxicity.

In conclusion, resveratrol administration not only inhibited platelet aggregation, reduced TXB$_2$ levels and GpIIb/IIIa receptor’s expression, but...
also increased the NKC's cytotoxicity. Resveratrol exhibited multiple mechanisms of action, resulting in an increased susceptibility of tumor cells to NKC. In addition, it has also been shown that other agents, such as pinene can also stimulate NK activity sufficiently. Thus, resveratrol can be used as an additional supplement to modulate the immune system and to inhibit platelet aggregation in thromboembolic episodes. Further clinical investigation in vivo could lead to specific concentrations that may maximize the beneficial effect of resveratrol.

References
17 Norseth T (1964) Nordisk Med 71, 375-377