

## **Resveratrol Diminishes Platelet Aggregation and Increases Susceptibility of K562 Tumor Cells to Natural Killer Cells**

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### **Abstract**

Platelet aggregation around migrating cancer cells protects them against the activity of natural killer cells (NKC). 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 B<sub>2</sub>(TXB<sub>2</sub>) 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 resveratrol. Resveratrol at a concentration of  $3 \times 10^{-3}$  M completely inhibited platelet aggregation ( $p < 0.05$ ). At the same concentration, it increased the NKCs cytotoxic activity at an average rate of  $319 \pm 34$ ,  $450 \pm 34$  and  $62 \pm 2.4\%$  ( $p < 0.05$ ) in the NKC/targets cells ratios of 12.5:1, 25:1 and 50:1, respectively. 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.

### **1. Introduction**

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 2,3. NKC's participate in the clearance of viral infection, especially in the innate immune response that occurs in the early phase of infection 4. Their actions include destruction of infected cells, secretion of inflammatory cytokines and interaction with dendritic cells 5. NKC's also destroy malignant and virus-infected cells by direct contact 6 and thus the migration of platelets around tumour cell protects them from NKC's lysis 7. 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 8. Platelets also secrete organic substances, such as TXA<sub>2</sub>, which is later metabolized to TXB<sub>2</sub> during the inflammation process 9 and microparticles which regulate the action of other blood cells like lymphocytes 10. 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 11. Polyphenols have been shown to possess various beneficial health properties 12. 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.

## **2. Materials and Methods**

### **2.1 Equipment**

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

### **2.2 Chemical and reagents**

Epinephrine (EPN), adenosine phosphate (ADP), arachidonic acid (ARA), platelet activating factor (PAF), thrombin (THR), resveratrol (R), phosphocreatine, creatine phosphokinase, acetylsalicylic acid and ginsolides A and B, Ficoll, phosphate-buffered saline and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). TXB<sub>2</sub> levels were estimated by the TXB<sub>2</sub> <sup>125</sup>I-DINOR-TXB<sub>2</sub> [125] radioimmuno assay kit (Isotop Company, Institute of Isotops Co. Ltd. Budapest, Hungary). Expression of GpIIb/IIIa receptors was measured by using the «A DIAflo Platelet GpIIb/IIIa Occupancy» kit (American Diagnostics, Inc. USA). The evaluation of NKC cytotoxicity was done using the kit NKTEST.

### **2.3 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)

#### **2.4 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 M), ADP (12 M), ARA (0.7 M), PAF (15 M) and THR (1 IU/ml). Five microliter from each agonist was added in 450 l of PRP for each measurement. Same measurements were repeated after incubation of each sample of 450 l PRP with 5 l addition of resveratrol ( $3 \times 10^{-3}$  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 TX B 2 production TXB2 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. TX B 2 was estimated in PRP before platelet aggregation and 5 min after its initiation, with and without administration of resveratrol ( $10^{-5}$  M to  $3 \times 10^{-3}$  M). 1.25 mg of indomethacin was used to stop

TX B 2 production by the arachidonic acid pathway enzyme. The samples were centrifuged at 5000 rpm/min for 5 min and supernatant was collected and processed as described previously.

#### **2.5 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  $3 \times 10^{-3}$  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 21. The isolated cells were then diluted in complete medium solution and their number was set at  $5 \times 10^6$  cells/ml by using the hemacytometer. The suspension contained the population of NKCs remained at room temperature till use.

#### **2.6 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  $10^5$  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 l. The samples were incubated for 2.5 h in a CO<sub>2</sub>

incubator. Cells' nuclei were labeled with red fluorescence by propidium iodide, in order to detect the apoptotic and necrotic TC. Estimation of cytotoxicity of NKC's was performed by flow cytometric analysis 22,23. Same measurements were repeated in presence of 50 l of resveratrol ( $10^{-5}$  M to  $3 \times 10^{-3}$  M) in the suspension of NKC/TC in the above-mentioned ratios before the incubation stage.

### 2.7 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).

## 3. Results and Discussion

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 ( $3 \times 10^{-3}$  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 inhibited 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 act 8,25,26. Furthermore, the number of GPIIb/IIIa receptors was significantly decreased after the addition of resveratrol ( $3 \times 10^{-3}$  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 27. 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 28, possibly resveratrol might influence mechanisms of immune cells' action through platelets and thus modulating the immune responses. Addition of resveratrol ( $3 \times 10^{-3}$  M) modified the NKC's 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 NKC's was engaged against TCs. One possible explanation was that resveratrol initiated the activating receptors of NKC's 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 29. Moreover, KIRs work

together with the lectin-like receptors CD94-NKG2C (an activating receptor) 30 .It has been shown that resveratrol act via NKG2D-dependent c-June-N-terminal kinase (JNK) and extracellular-regulated kinase (ERK-1/2 pathways) 31 In this manner, NKC's become more active and activate their killing mechanisms directly against TCs. Resveratrol also exerts partial agonist activity to aryl hydrocarbon receptor (AhR) 32 . In conclusion, resveratrol administration not only inhibited platelet aggregation, reduced TXB<sub>2</sub> levels and GpIIb/IIIa receptor's expression, but also increased the NKC's cytotoxicity. Resveratrol exhibited multiple mechanisms of action, resulting in increase of the susceptibility of tumor cells to NKC's. In addition, it has also been shown that other agents, such as pinene can also stimulate NK activity sufficiently 35. 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.

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