Inhibition of platelet aggregation and immunomodulation of NK lymphocytes by administration of ascorbic acid

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Platelet aggregation around migrating tumor cells offers protection against the cytotoxic activity of the natural killers cells (NKC). The ascorbic acid in $3 \times 10^{-3} \text{M}$ concentration completely inhibited platelet aggregation, decreased thromboxane B$_2$ levels, and inhibited the expression of platelet membranic receptor GpIIb/IIIa in non stimulated platelets, and increased the NKC cytotoxicity in an average rate of 105, 61, and 285% in the NKC/targets cells ratios 12.5:1, 25:1 and 50:1 respectively. The results suggest the role of ascorbic acid in increasing the susceptibility of tumor cells to NKC; the ascorbic acid could be used as part of a multidrug therapy to treat diseases which up to now have been treated only through chemotherapy.

Keywords: Ascorbic acid, Natural killer cells, Platelet glycoprotein GpIIb-IIIa complex, Thromboxane B$_2$

The process of platelet aggregation is mediated by the binding of fibrinogen to GpIIb/IIIa, a glycoprotein which is a platelet surface receptor. Fibrinogen binds to this activated receptor and this binding plays an important role in the formation of thrombus. While GpIIb/IIIa inhibitors block fibrinogen-platelet binding, stimulation of other functionally important platelet receptors may still occur. Blocking the GpIIb/IIIa receptor prevents platelet aggregation but not activation and the subsequent effect on other platelet pathways is largely unknown. Besides the coagulation process, it is considered that platelets participate in inflammatory reactions via secretion of drastic substances such as thromboxane A$_2$ (TXA$_2$). TXA$_2$, secreted by platelets during platelet stimulation and aggregation is totally metabolized to TXB$_2$, which constitutes a molecule of stable structure but no aggregative ability.

During platelet stimulation by different agonists such as thrombin, along with reactive substances platelet-derived microparticles (PMP) are secreted by these cells and act as message transmitters towards other blood cells like lymphocytes, regulating their action. In this way, they are involved in the process of immune response in the pathogenesis of neoplasmatic diseases, but their mechanism of action still remains unknown. Studies where antiplatelet drugs were administered in patients with neoplasmatic diseases showed that these drugs could have a possible action in angiogenesis, tumor development and metastases of the involved patients.

Natural killer cells (NKC) basic action is to destroy viruses, bacteria and cancer cells. Platelets aggregation around migrating tumor cells directly protect them from NKC lysis since these cells exert their cytotoxic activity by direct contact. NKC play a major role in the rejection of tumors, and their functionally is of great prognostic value in the treatment of malignacies.

Substances with antioxidant properties in platelets and lymphocytes functionality have been shown to be potent inhibitors of platelet activation, while they induce cytotoxicity of NKC against cancer cells.

The objective of this study is to evaluate the ability of ascorbic acid (AA) to increase the susceptibility of tumor cells to NKC, through the modulation of platelet aggregation and NKC cytotoxic activity, in a series of in vitro and ex vivo experiments.
Materials and Methods

Chemicals and reagents—Epinephrine (EPN), adenosine phosphate (ADP), arachidonic acid (ARA), platelet activating factor (PAF), thrombin (THR), AA, phosphocreatine, creatine phosphokinase, acetylsalicylic acid, 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 kit TXB$_2$/2,3-DINOR-TXB$_2$[125I] radioimmune assay KIT (Isotop company, Institute of Isotops Co. Ltd. Budapest, Hungary) and expression of GpIIb/IIIa receptors was measured by using the «ADIAflo Platelet GpIIb/IIIa Occupancy» kit of American Diagnostics, Inc. USA. Finally, the kit used for the evaluation of NKC cytotoxicity was NKTEST of ORPEGEN Pharma, Germany.

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

Platelet rich plasma (PRP) preparation—Whole blood (20 ml) was taken from each of 28 healthy volunteers by free flow and transferred in plastic tubes containing 3% citric nitrate. Blood samples were then centrifuged at 900 rpm for 10 min and platelet rich plasma (PRP) was isolated as supernatant. For the calibration of the aggregometer, the rest of the blood samples were centrifuged again at 3100 rpm for 15 min and platelet poor plasma (PPP) was collected as supernatant. Platelets concentration was fixed in 2.5×10$^9$ cells/ml by Brecher-Cronkite’s method.$^{13}$

Platelet aggregation—Platelet aggregation was performed into the aggregometer’s cuvettes using the following platelet stimulators in concentrations that cause maximum non-reversible platelet aggregation: epinephrine (EPN, 5 $\mu$M), adenosine phosphate (ADP, 12 $\mu$M), arachidonic acid (ARA, 0.7 $\mu$M), platelet activating factor (PAF, 15 $\mu$M) and thrombin (THR, 1 IU/ml). The volume of the solution of each one of the above substances added in 450 $\mu$l of PRP was 5 $\mu$l for each measurement. Same measurements were repeated after incubation of each sample of 450 $\mu$l PRP by 5 $\mu$l addition of AA in concentrations ranging from 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.

Platelet TXB$_2$ production—TXB$_2$ was estimated in PRP before platelet aggregation and 5 min after its initiation, with and without administration of AA in the concentrations mentioned above. In order to stop TXB$_2$ production by the arachidonic acid pathway enzymes,$^{14}$ in each PRP sample, 1.25 mg of indomethacin was administered after the completion of platelet aggregation. The samples were centrifuged at 5000 rpm/min for 5 min and supernatant was collected and processed as per Powell.$^{15}$ Radioactivity of each sample was measured by $\gamma$-counter and the result was expressed in pgr TXB$_2$/ml.

Platelet GpIIb/IIIa receptors—Expression of GpIIb/IIIa receptor per platelet was estimated by flow cytometric analysis.$^{16,17}$ 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 AA in the concentrations mentioned above.

Isolation of peripheral blood mononuclear cells (PBMC) —Whole blood (20 ml) was collected from 12 volunteers and transferred into tubes that contained heparin as anticoagulant. Isolation of PBMC was performed as previously described.$^{18}$ The isolated cells were diluted in complete medium solution and their number was set at 5×10$^6$ cells/ml by the use of hemocytometer. The suspension contained the population of NKC remained at room temperature till use.

NKC functionality—For evaluation of NKC functionality, chronic myeloid leukemia cells from K562 cell line were used as target cells (TC). After dilution of TC in complete medium and setting their concentration to 10$^5$cells/ml, their membranes were labeled with green fluorescence by fluorescein isothiocyanate and the suspensions of NKC and TC were mixed in NKC/TC ratios of 12.5:1, 25:1 and 50:1 in a final volume of 200 $\mu$l. The samples were incubated for 150 min in a CO$_2$ incubator, cells’ nuclei were labeled with red fluorescence by propidium iodide. In order to detect the apoptotic and necrotic TC, estimation of cytotoxicity of NKC was performed by flow cytometric analysis.$^{19,20}$ Same measurements were repeated after administration of 50 $\mu$l of AA in concentrations of 3×10$^{-3}$, 10$^{-3}$M, 10$^{-4}$ and 10$^{-5}$M in the suspension of NKC/TC in the above mentioned ratios before the incubation stage.
Statistical analysis—Data are expressed as mean ± SD. Results were statistically evaluated by Student’s t-test and value of $P<0.05$ was considered statistically significant (SPSS version 16.0, Chicago, USA).

Results

Platelet experiments

Platelet aggregation—Ascorbic acid in $3 \times 10^{-3}$M concentration completely inhibited platelet aggregation induced by all platelet stimulators used (Table 1). Platelet aggregation was slightly inhibited at $10^{-3}$M concentration by all platelet stimulators. There was no inhibitory effect on platelet aggregation in the other two concentrations ($10^{-4}$ and $10^{-5}$M).

Platelet $\text{TxB}_2$ production—Levels of $\text{TxB}_2$ secreted by stimulated platelets were significantly decreased after administration of $3 \times 10^{-3}$M AA ($P<0.05$); (Fig. 1). In all others concentrations there was no statistical difference in levels of $\text{TxB}_2$ (results not shown).

Table 1—Inhibition (%) of platelet aggregation by various doses of ascorbic acid

<table>
<thead>
<tr>
<th>Ascorbic acid concentration (M)</th>
<th>EPN</th>
<th>PAF</th>
<th>ADP</th>
<th>ARA</th>
<th>THR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>9±2</td>
<td>15±2</td>
<td>12±4</td>
<td>6±1</td>
<td>3±1</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>57±8</td>
<td>73±3</td>
<td>65±3</td>
<td>60±3</td>
<td>50±2</td>
</tr>
<tr>
<td>$3 \times 10^{-3}$</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

EPN=epinephrine; ADP=adenosine phosphate; ARA=arachidonic acid; PAF=platelet activating factor; THR=thrombin

Fig. 1—Levels of $\text{TxB}_2$ secreted by stimulated platelets before and after administration of $3 \times 10^{-3}$ M ascorbic acid. [$\text{TxB}_2$ was estimated in PRP with and without administration of ascorbic acid. Radioactivity of each sample was measured by $\gamma$-counter and the result was expressed in pgr $\text{TxB}_2$/ml. Values are mean ± SD. *significantly different]

Platelet GpIIb/IIIa receptors—AA in $3 \times 10^{-3}$M concentration significantly inhibited (from 90138±4562 to 1408±112 receptors per platelet) platelet GpIIb/IIIa receptor’s expression ($P<0.05$), while no statistical alteration was observed in the other three concentrations of AA.

NKC experiments

Concentration $3 \times 10^{-3}$M increased NKC cytotoxicity in an average rate of 105, 61, and 285% in the ratios 12.5:1, 25:1, and 50:1 respectively (Fig. 2). The NK cytotoxicity in the other three concentrations of AA remained stable and statistically not significant. When the suspension of TC was incubated only with AA (without NKC), cytotoxicity was 7.5% in an average rate.

Discussion

The ascorbic acid completely inhibited platelet aggregation at $3 \times 10^{-3} \text{M}$ concentration and enhanced the activity of the NK lymphocytes in vitro. Thus, this lead to an increase of NKC ability to exert their cytotoxic activity on the migrating tumor cells.

$\text{TxB}_2$ is the final stable metabolite of the unstable $\text{TXA}_2$, which is produced by the platelet enzymic COX system via biochemical reactions where oxygen free radicals production takes place. Inhibition of cyclooxygenase (COX) action by administration of aspirin or other non steroid anti-inflammatory drugs...
(NSAID) stops TXA2 synthesis\textsuperscript{22,23}. In the present study TXB2 production was increased more when platelets were stimulated by THR, ARA and PAF than EPN and ADP before platelet aggregation. When AA was added in the same experiment, the TXB2 production was decreased in all samples which were stimulated by all five antagonists. It was observed that when platelet aggregation increased, TXB2 quantity also increased and vice-versa after the administration of AA. The above result indicates that AA could play a regulatory role in all five antagonists.

The results showed that AA possibly acts as an anti-inflammatory factor inhibiting COX action. Anti-platelet action of AA is possibly due to its antioxidant properties, given that other substances with similar properties act as platelet antagonists by inhibiting COX activity\textsuperscript{24,25}. Inhibition of COX by antioxidant substances is caused by the scavenging of oxygen free radicals, which results in decrease of TXA2 production, inhibition of platelet activation, and GpIib/IIia membrane receptor’s expression\textsuperscript{1}. In the present study GpIib/IIia receptors were decreased significantly after the addition of AA in non stimulated platelets, suggesting that the blocking of the GpIib/IIia receptor prevents platelet aggregation. This glycoprotein is considered a major biological marker in vascular function issues\textsuperscript{2}. Thus, there is great interest in the clinical development of agents that can bind to platelet GpIib/IIia, block fibrinogen binding and be used in the prevention and management of thrombotic disease states. Since platelets are regulators of immune cells’ action during the immune response\textsuperscript{26}, it is possible that AA influences the mechanisms of immune cells’ action though platelets and by this way modulates immune responses.

Data from NKC experiments showed that the addition of AA modified the NKC in a way that caused the increase of functionality against K562 TC. This happened possibly because AA may have initiated the activating receptors of NKC and increased the motility of these cells. By this way NK cells become more active and activate their killing mechanisms directly against TC. Moreover, it is important to notice that AA wasn’t toxic against K562 cancer cells, and it didn’t cause any significant apoptosis to the population of NKC. The immunomodulating action of this substance is significant on the ratios 12.5:1 and 25:1 where a small number of NKC is involved against TC. This data show that AA modified NKC behavior against TCs, especially when a small number of lymphocyte population faces large numbers of TCs. It is known that in many pathological conditions like autoimmune disease, cancer, AIDS and diabetes type I, NKC cytotoxicity is significantly reduced\textsuperscript{27,28,29}. Although the mechanism of action of the NKC remains unknown today, the fact that only one cell alone can destroy 27 cancer cells before its death proves that these cells are the brain of the immune system for the human organism\textsuperscript{30}.

In summary, AA administration inhibited platelet aggregation, reduced TXB2 levels and GpIib/IIia receptor’s expression and enhanced the NKC cytotoxicity. Ascorbic acid exhibited multiple mechanisms of action which resulted in increase of the susceptibility of tumor cells to NKC. AA has no side effects since it can be excreted from the human body after its administration. Thus, AA could be used as part of a multidrug therapy to treat diseases which up to now have been treated only through chemotherapy.

**Conflict of interest**

The authors declare no conflict of interest.

**References**


