Indian Journal of Experimental Biology
Vol. 49, December 2011, pp. 904-908

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

Ioannis K. Toliopoulos[1,2,*], Yannis V. Simos[1,2], Telemachos A. Daskalou[1,3], Ioannis I. Verginadis[1,2], Angelos M. Evangelou[1] & Spyros C. Karkabounas[1]

[1]Laboratory of Physiology, Faculty of Medicine, University of Ioannina, Ioannina 45110, Greece

[2]Konstantinion Research Center of Molecular Medicine and Biotechnology (Non Profit Foundation), Thessaloniki, Greece

[3]Melission Amalia Fleming General Hospital, Athens, Greece

Received 18 May 2011; revised 20 July 2011

Platelets aggregation around migrating tumor cells offers protection against the cytotoxic activity of the natural killers cells (NKC). The ascorbic acid in 3×10^{-3}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[1]. 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[2]. 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[3, 4].

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[5]. 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[6].

Natural killer cells (NKC) basic action is to destroy viruses, bacteria and cancer cells[7]. Platelets aggregation around migrating tumor cells directly protect them from NKC lysis[8] since these cells exert their cytotoxic activity by direct contact[9]. 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[10,11], while they induce cytotoxicity of NKC against cancer cells[12].

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.

*Correspondent author
Telephone: +30-2310 838411
Fax: +30-2310 838411
E-mail: johntolio@hotmail.com

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[^{125}] 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 γ-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 one 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 μM), adenosine phosphate (ADP, 12 μM), arachidonic acid (ARA, 0.7 μM), platelet activating factor (PAF, 15 μM) and thrombin (THR, 1 IU/ml). The volume of the solution of each one of the above substances added in 450 μl of PRP was 5 μl for each measurement. Same measurements were repeated after incubation of each sample of 450 μl PRP by 5 μl 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 γ-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 hematocytometer. 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 10^5cells/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 μl. 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 μl 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 3×10^{-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 TXB_2 production—Levels of TXB_2 secreted by stimulated platelets were significantly decreased after administration of 3×10^{-3}M AA ($P<0.05$); (Fig. 1). In all others concentrations there was no statistical difference in levels of TXB_2 (results not shown).

Table 1—Inhibition (%) of platelet aggregation by various doses of ascorbic acid [Values are mean ±SD]

Ascorbic acid concentration (M)	Stimulators				
	EPN	PAF	ADP	ARA	THR
10^{-5}	0	0	0	0	0
10^{-4}	9±2	15±2	12±4	6±1	3±1
10^{-3}	57±8	73±3	65±3	60±3	50±2
3×10^{-3}	100	100	100	100	100

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

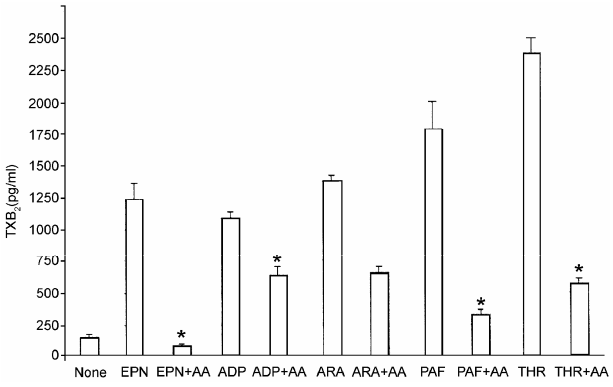

Fig. 1—Levels of TXB_2 secreted by stimulated platelets before and after administration of 3×10^{-3} M ascorbic acid. [TXB_2 was estimated in PRP with and without administration of ascorbic acid. Radioactivity of each sample was measured by γ-counter and the result was expressed in pgr TXB_2/ml. Values are mean ± SD. *significantly different]

Platelet GpIIb/IIIa receptors— AA in 3×10^{-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 3×10^{-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 3×10^{-3} 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.

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[21]. Inhibition of cycloxygenase (COX) action by administration of aspirin or other non steroid anti-inflammatory drugs

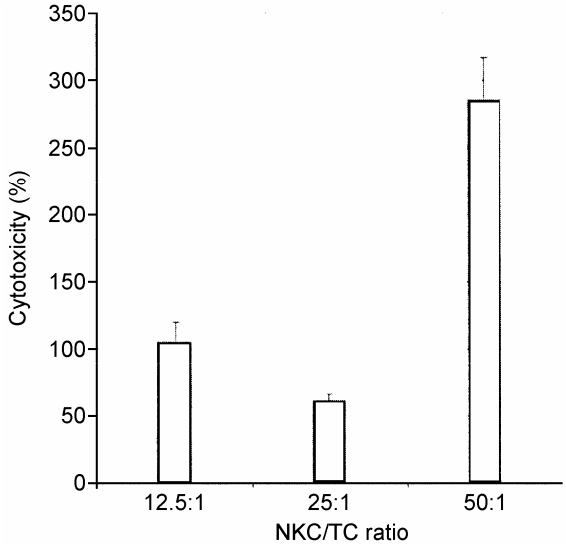

Fig. 2—NKC cytotoxicity (%) after the administration of ascorbic acid in 3×10^{-3}M concentration in the ratios 12.5:1, 25:1, 50:1. [For the evaluation of NKC functionality, chronic myeloid leukemia cells from K562 cell line were used as target cells. Estimation of cytotoxicity of NKC was performed by flow cytometric analysis. Values are mean ± SD]

(NSAID) stops TXA_2 sythesis[22,23]. In the present study TXB_2 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 TXB_2 production was decreased in all samples which were stimulated by all five antagonists. It was observed that when platelet aggregation increased, TXB_2 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[24,25]. Inhibition of COX by antioxidant substances is caused by the scavenging of oxygen free radicals, which results in decrease of TXA_2 production, inhibition of platelet activation, and GpIIb/IIIa membrane receptor's expression[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[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[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[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[30].

In summary, AA administration inhibited platelet aggregation, reduced TXB_2 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

1 Chang SJ, Lin JS & Chen HH, Alpha-tocopherol downregulates the expression of GPIIb promoter in HELA cells, *Free Radic Biol Med*, 28 (2000) 202.

2 Chakrabarti S, Clutton P, Varghese S, Cox D, Mascelli MA & Freedman JE, Glycoprotein IIb/IIIa inhibition enhances platelet nitric oxide release, *Thromb Res*, 113 (2004) 225.

3 Benedetto C, McDonald-Gibson RC, Nigam S & Staler TF, *Prostaglandins and related substances. A practical approach*. (IRL Press Ltd, Oxford, UK), 1987.

4 Palrono C & Peskar BA, *Radioimmuroassay in basic and clinical pharmacology. Handbook of experimenta lpharmacology. Vol. 82*. (Springer-Verlag, Berlin), 1987.

5 Wazna E, Platelet-mediated regulation of immunity, *Postepy Hig Med Dosw*, 60 (2006) 265.

6 Mousa SA, Antithrombotics in thrombosis and cancer, *Hamostaseologie*, 25 (2005) 380.

7 Dokun AO, Kim S, Smith HR, Kang HS,Chu DT & Yokoyama WM, Specific and nonspecific NK cell activation during virus infection, *Nat Immunol*, 2 (2001) 951.

8 Nieswandt B, Hafner M, Echtenacher B & Mannel DN, Lysis of Tumor cells by Natural Killers Cells in mice is impeded in platelets, *Cancer Res*, 59 (1999) 1295.

9 Storkus WJ & Dawson JR, Target structures involved in natural killing (NK): Characteristics, distribution, and candidate molecules, *Crit Rev Immunol*, 10 (1991) 393.

10 Daskalou T, Karamouzis M & Liaros G, Metabolites of arachidonic acid in activating platelets and their estimation by radionuclide techniques, *Hell J Nucl Med*, 9 (2006) 49.

11 Wayne RL & Mahinda YA, Cardioprotective actions of grape polyphenols, *Nutrition Res*, 28 (2008) 729.

12 Evangelou A, Kalpousos G, Karkabounas S, Liasko R, Nonni A, Stefanou D & Kallistratos G, Dose-related preventive and therapeutic effects of antioxidants-anticarcinogens on experimentally induced malignant tumors in Wistar rats, *Cancer Lett*, 115 (1997) 105.

13 Norseth T, Thrombocyte count in the capillary blood. A comparison of the Brecher-Cronkite and Bjoerkman methods, *Nord Med*, 71 (1964) 375.

14 Patrono C, Ciabattoni G, Pugliese F, Pinca E, Castrucci G, De Salvo A, Satta A & Parachini M, Radioimmunoassay of serum thromboxane B_2, a simple method of assessing pharmacologic effects on platelet function, *Adv Prostaglandin Thromboxane Res*, 6 (1980) 187.

15 Powell WS, Rapid extraction of oxygenated metabolites of arachidonic acid from biological samples using octadecylsilyl silica, *Prostaglandins*, 20 (1980) 947.

16 Hezard N, Metz D, Nazeyrollas P, Nguyen P, Simon G, Daliphard S, Droulle C, Elaerts J & Potron G, Free and total platelet glycoprotein IIb/IIIa measurement in whole blood by quantitative flow cytometry during and after infusion of c7E3 Fab in patients undergoing PTCA, *Thromb Haemost*, 81 (1999) 869.

17 Quinn M, Deering A, Stewart M, Cox D, Foley B & Fitzgerald D, Quantifying GPIIb/IIIa receptor binding using 2 monoclonal antibodies, discriminating abciximab and small molecular weight antagonists, *Circulation*, 99 (1999) 2231.

18 Neri S, Mariani E, Meneghetti A, Cattini L & Facchini A, Calcein-acetyoxymethyl cytotoxicity assay, standardization of a method allowing additional analyses on recovered effector cells and supernatants, *Clin Diagn Lab Immunol*, 8 (2001) 1131.

19 Toliopoulos JI, *The role of NKC lymphocytes and substances with antioxidant capacity in cancer*. Ph.D. thesis, University of Ioannina, Ioannina, 2007.

20 Henney CS, Kuribayashi K, Kern DE & Gillis S, Interleukin-2 augments natural killer cell activity, *Nature*, 291 (1981) 335.

21 Suttnar J, Masova L, Scheiner T, Sorelova V & Dyr JE, Role of free radicals in blood platelet activation, *Cas Lek Cesk*, 141 (2002) 47.

22 Fitzpatrick FA, Cyclooxygenase enzymes, regulation and function, *Curr Pharm Des*, 10 (2004) 577.

23 Tries S, Laufer S, Radziwon P & Breddin HK, Antithrombotic and platelet function inhibiting effects of ML3000, a new antiinflammatory drug with Cox/5-LOX inhibitory activity, *Inflamm Res*, 51 (2002) 129.

24 Simmler C, Antheaume C & Lobstein A, Antioxidant Biomarkers from Vanda coerulea Stems Reduce Irradiated HaCaT PGE-2 Production as a Result of COX-2 Inhibition, *PLoS One*, 5 (2010) e13713.

25 Granados-Soto V, Pleiotropic effects of resveratrol, *Drug News Perspect*, 16 (2003) 299.

26 Hundelshausen P & Weber C, Platelets as immune cells, bridging inflammation and cardiovascular disease, *Circ Res*, 100 (2007) 27.

27 Johann PD, Vaegler M, Gieseke F, Mang P, Armeanu-Ebinger S, Kluba T, Handgretinger R & Müller I, Tumour stromal cells derived from paediatric malignancies display MSC-like properties and impair NK cell cytotoxicity *BMC Cancer*, 10 (2010) 501.

28 Chen G, Han G, Wang J, Wang R, Xu R, Shen B, Qian J & Li Y, Natural killer cells modulate overt autoimmunity to homeostasis in nonobese diabetic mice after anti-CD3 F(ab')2 antibody treatment through secreting transforming growth factor-beta, *Am J Pathol*, 175 (2009) 1086.

29 Strbo N, de Armas L, Liu H, Kolber MA, Lichtenheld M & Pahwa S, IL-21 augments natural killer effector functions in chronically HIV-infected individuals, *AIDS*, 22 (2008) 1551.

30 Whiteside TL & Herberman RB, Role of human natural killer cells in health and disease, *Clin Diagn Lab Immunol*, 1 (1994) 125.