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Oxidative Stress and Platelet Dysfunction

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Abstract

The redox state modulates the functioning of many cells, including platelets. Oxidative stress is associated with many diseases, such as cancer, diabetes, cardiovascular and neurodegenerative diseases as well as hemolytic anemias. Oxidative stress in platelets may lead to their hyper activation and excess clot formation, thus, Thereby to thromboembolic complications that are a common co-morbidity of most of these diseases. We hereby review evidence that support the possibility that the platelet redox state might present a therapeutic target in cases of either hypo- or hyper platelet activation, using oxidants or antioxidants, respectively. Measurement of the platelet redox state by flow cytometry might direct the mode of treatment and serve for evaluation of its efficacy.

Keywords: Antioxidants; Flow cytometry; Oxidative stress; Platelets; Thromboembolic complications

Oxidative Stress in Human Pathology

The redox equilibrium of cells represents the balance between oxidative agents such as Reactive Oxygen Species (ROS) and reactive nitrogen species, and anti-oxidative systems that either scavenge these species or ameliorate their effects. This balance is crucial for physiological functioning of cells [1], e.g., signal transduction pathways [2], and as such it is highly regulated [3]. An increased oxidative activity that is not counter-balanced by antioxidative activities, results in oxidative stress. Since the oxidizing species interact with cellular components, when they are present in excess they are cytotoxic, and thus result in malfunctioning of vital organs. Oxidative stress may result from excess ROS production or shortage of antioxidants due to environmental insults (pollution, radiation, etc.) and the pathological conditions of many diseases including cancer, diabetes, cardiovascular [4], neurodegenerative diseases [5] as well as aging [6]. Oxidative stress is the secondary outcome of these diseases, exacerbating their symptoms.

We have studied oxidative stress in hemolytic anemias [7]. These anemias are characterized by augmented destruction (hemolysis) of mature Red Blood Cells (RBC) and their precursors that is not balanced by compensatory overproduction of RBC. Among these diseases are: (A) the hemoglobinopathies–caused by hereditary mutations in the globin genes, leading to insufficient production (thalassemia) or production of aborted (sickle cell disease) globin chains [8]. (B) Hereditary spherocytosis–caused by mutations in the genes of membrane proteins leading to spherical rather than the biconcave shape of the RBC [9]. (C) Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency–caused by point mutations in the G6PD gene. G6PD is a key enzyme of the pentose pathway (hexose monophosphate shunt) which supplies Nicotinamide Adenine Dinucleotide Phosphate (NADPH)–a reducing agent that is important for the regulation of the redox state, especially in RBC [10]. Patients exhibit hemolytic anemia in response to infection and certain medications or foods. (D) Paroxysmal Nocturnal Hemoglobinuria (PNH)–caused by an acquired somatic mutation in the phosphatidylinositol glycan complementation class a gene. This gene encodes the enzyme responsible for the first step in the production of the glycosylphosphotidylinositol anchor, by which various proteins are linked to the plasma membrane. The mutation leads to lack/deficiency of membrane proteins (CD55 and CD59) that protect cells from binding of the activated plasma complement. In RBC, this leads to extravascular hemolysis, and in platelets–to venous thrombosis [11]. (E) Congenital dyserythropoietic anemias–a heterogeneous group of rare diseases in which the anemia is predominantly caused by ineffective erythropoiesis and distinct morphological abnormalities of erythroid precursor cells in the bone marrow (dyserythropoiesis) [12].

Although oxidative stress is not the primary etiology and symptom of most of these diseases, it is involved in many of their symptoms, including anemia and thromboembolic complications [7]. Oxidative stress in these diseases results from the generation of excess ROS due to: (A) Intracellular degradation of abnormal hemoglobins (in the hemoglobinopathies), leading to the production of hemichromes. (B) Iron-overload caused by frequent blood transfusions and increased iron uptake in the gastrointestinal tract. The latter is due to continuous, yet ineffective, attempt to accelerate erythropoiesis to overcome the anemia. The increased iron uptake is mediated by decreased production of the peptide hepcidin – the main regulator of iron uptake and distribution [13]. Iron-overload increases ROS generation by catalyzing the Fenton, Haber-Weiss biochemical reactions [1,14].

Oxidative Stress and Abnormal Platelet Function

Platelets (thrombocytes) are a major player in preventing bleeding (hemostasis). Mechanical damage to blood vessels initiates localized responses that engage platelets: adhesion to the endothelium, activation and aggregation, leading to clot (thrombus) formation (coagulation) and repair. These platelet activities involve the generation of intracellular chemical signals that are initiated through specific surface receptors (e.g. integrins) (for review see [15]). Defects in platelet function may cause defective clot formation: Hypo-activity-delays it, resulting in life-threatening bleeding (hemorrhage),

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while hyper-activity-increased it, resulting in obstructive clotting (thrombosis). Thus, a therapeutic intervention of opposing effects may be required, depending on the clinical situation. Platelet malfunction may be associated with their anomalous numbers, low (thrombocytopenia) or high (thrombocytosis), due to abnormal production or destruction, respectively. Alternatively, irrespective of their number, their malfunction may be due to inherited or acquired defects.

Platelet functioning depends on their redox state. Platelets have an inherent ability to produce ROS by various pathways- as a byproduct of the respiratory in their mitochondria [16] and by the NADH/NADPH oxidase [17] ([for a detailed review see [18]) produced mainly in the pentose cycle. ROS, along with nitric oxide, adenosine and prostacyclins, may play a profound role in the regulation of platelet activities [19]. Many studies suggested that their functioning during clot formation involves increased in ROS, e.g., ROS production is increased by platelet activators such as thrombin [20-22].

Oxidative stress in platelets may result in two pathological outcomes: Toxicity, as in other cell types [23], resulting in thrombocytopenia and bleeding. It may also be involved in their hyperactivation resulting in excess clot formation leading to thromboembolic complications. Exemplifying the latter are the effects of hydrogen peroxide that stimulates their oxidative stress [24]. These effects include platelet activation depending on arachidonic acid and collagen [25] as well as by thrombin and ADP [26-28] and by inducing tyrosine phosphorylation of the platelet αIIbβ3, an independent platelet activation mechanism, enhancing their aggregation [29], as well as through scavenging of the platelet or endothelium-derived nitric oxide and thereby decreasing its aggregation-inhibiting effect [30]. Superoxide can also contribute to late thrombus growth *via* increasing the bioavailability of ADP and subsequently, recruiting additional platelets [28].

Since platelets are not known to suffer from a specific inherent pathology of their redox regulation, it is reasonable to attribute, at least part of, their oxidative stress to continuous exposure to oxidative insults from extra-platelet sources–neighboring cells, e.g., the vascular endothelium, and components of their environment, the plasma. We have shown that incubation of normal platelets with plasma from thalassemia patients, rather than with normal plasma, resulted in their oxidative stress and activation [24]. Among the various components in the plasma that may induce this effect are iron-containing compounds such as transferrin-bound iron and non-transferrin-bound iron, ferritin, heme or hemoglobin, all of which increase in the plasma of thalassemia patients [31,32]. Incubation of platelets with iron (ferric ammonium citrate), heme (hemin or heme arginate) or hemoglobin stimulated their oxidative stress. The involvement of iron of these compounds could be from the ability of the iron-chelator Desferoxamin to inhibit the effect of thalassemia plasma on the increase in the platelets' ROS [24].

We have also shown that incubation of normal platelets with thalassemic RBC resulted in their oxidative stress. Normal RBC had no such effect on platelets unless the RBC was treated with hydrogen peroxide [33]. These results suggest that thalassemic RBC, having a higher than normal ROS level, mediate oxidative stress in platelets directly, probably by contact or close proximity (Fibach, in press). These results are in agreement with studies showing that platelets could be activated by ROS generated by neighboring cells such as RBC, neutrophils [34,35], vascular wall endothelial cells, vascular smooth muscle cells, and fibroblasts [18].

RBC might also affect platelets indirectly by a variety of mechanisms: (A) Release of iron-containing oxidants into the plasma [25,36,37], as mentioned above. (B) The release of ROS, such as superoxide anions, may cause oxidation of low-density lipoprotein [38], which, in turn, might activate platelets [39]. (C) Exposure or shedding of phosphatidylserine moieties, which act as a procoagulant that amplifies the generation of thrombin and thus initiates platelet activation [40]. Thalassemic RBC have been shown to carry and shed higher than normal levels of external phosphatidylserine [41].

The Platelet Redox Balance as a Therapeutic Target

The involvement of the platelet redox balance in hemostasis suggests that it can serve as a therapeutic target.

Oxidants and antioxidants may modulate platelet function by enhancing/reducing the oxidative status of platelets or other cells (e.g., RBC) that may in turn affect platelets. Examples include N-Acetyl Cysteine (NAC) and vitamin C [33,42]. Both agents inhibit platelet aggregation *in vivo* [43,44].

Dietary n-3 polyunsaturated fatty acid supplementation positively decreased the level of superoxide and lipid peroxidation in platelets of diabetic patients with hypertension [45,46]. Oral N-acetylcysteine has a potential to reduce atherothrombotic risk in type 2 diabetics through normalization of intra-platelet glutathione concentration [47].

Measurement of OS in Platelets

Several methods measure end-points of the platelet activation cascade but not the exact mechanisms underlying it. Since oxidative stress in platelets plays a profound role in their pathophysiology, its measurement might be helpful in revealing the etiology and for evaluating of the efficacy of anti-platelet targeted therapies [48].

Measurement of redox state parameters in cells and in body fluids, such as the blood plasma, can be accomplished by various methods [14]. In platelets, ROS have been measured by various techniques, using spectrophotometry [49], chemiluminescence, electron spin resonance and spin-trapping [50]. These measurements, however, are not a common practice in the clinic. A major limitation is the inadequacy of the techniques for routine clinical laboratory use. We have measured redox parameters in platelets by flow cytometry [33,51], a common methodology in the clinical setting. For measuring cellular oxidative stress parameters various fluorescence probes, such as 2'7'-Di Chlorodihydro Fluorescindiacetate (DCFDA) are employed [52]. This no fluorescent compound is taken up by cells, undergoes esterification, and gets trapped in the cells as 2'7'-Dichlorodihydrofluorescin (DCFH). ROS, mainly peroxides, oxidize it to the fluorescent derivative 2'7'-dichloro-Dihydrofluorescein (DCF). The fluorescence emitted by the cells is proportional to their ROS content. Exploiting of differences between cells in their size, granularity and/or expression of specific surface

markers permits simultaneous analysis of various blood cell types [53].

Several important considerations should, however, be taken when using this methodology: (A) ROS are extremely short-lived; analyses should be performed, therefore, on fresh samples. (B) The probes are usually not specific to a specific type of ROS-a limitation for studying a particular ROS, but not necessarily for the general assessment of oxidative stress. (C) As for measuring DCF fluorescence, intracellular DCFH concentration depends on the experimental setting: the concentration of DCFDA added the medium composition and the conditions (e.g., the temperature) of incubation. But, it also depends on the extent of DCFDA uptake by the cells and its esterification to DCFH – parameters that may vary in platelets under different conditions (activated *vs*. inactivated, pathological *vs*. normal). To overcome these caveats, we introduced a protocol that measures the kinetics of the change in the DCF fluorescence. Cells are pulsed with DCFDA for 15 min. washed thoroughly to remove exogenous DCFDA, and then re-incubated in DCFDA-free medium. Measuring the cellular fluorescence at different time points yields the kinetics of ROS generation.

The applicability of the method for measuring of platelet ROS was validated by the increased fluorescence following treatment of platelets with the ROS-generating agent peroxide, and with the catalase inhibitor sodium azide, and by the decreased fluorescence observed after treatment with the ROS scavenger NAC. When normal platelets were compared to platelets from β-thalassemia patients, both the basal fluorescence and its kinetics were higher in the latter, confirming that thalassemic platelets are under oxidative stress.

Conclusion

The redox state modulates the functioning of many cells, including platelets. Oxidative stress is associated with many diseases. Among other cells, platelets in these diseases display oxidative stress, leading to their hyper activation, excess clot formation and development of thromboembolic complications which are a co-morbidity characterizing many diseases.

We suggest that the platelet redox state might be a therapeutic target in cases of either hypo- or hyper platelet activation, using oxidants and antioxidants, respectively. Measurement of the platelet redox state by flow cytometry might direct the mode of treatment and serve for evaluation of its efficacy.

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