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The Involvement of Reactive Oxygen Species in the Development of Unstable Angina

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Abstract

Inflammatory process has been found to play an important role in the pathogenesis of coronary heart disease (CHD). The purpose of our study was to investigate the reactive oxygen species of peripheral neutrophils during the development of angina in the stable and the unstable stage. We have investigated the phagocytosis and the superoxide anion release after in vitro stimulation of neutrophils. The neutrophils phagocytic activity was evaluated as % of cells that engulf zymosan particles and the superoxide anion release was evaluated as cytochrome reduction test. Our results indicate that the concentration of peripheral neutrophils is statistically higher in the unstable stage of the angina compared with the normal values count or with the stable angina patients. The superoxide anion release of neutrophils isolated from unstable angina patients is significantly enhanced compared with normal subjects or with the values observed in the stable stage of the angina. The unstable angina presents alteration of some cellular immune parameters that indicate an inflammatory syndrome associated with an increased risk of CHD, having also a prediction value for the plaque instability.

Keywords: neutrophil, superoxide anion, phagocytosis, unstable angina

Introduction

Coronary artery disease (CAD) is related to endothelial dysfunction, inflammation and atherosclerosis. CAD includes a spectrum of conditions, from stable angina to acute coronary syndromes (unstable angina and myocardial infarction), the last ones representing high-risk processes with severe life-threatening thrombotic events [1]. Increasing evidence supports the involvement of inflammation in acute phase of CAD [2].

Total or near-total sudden arterial occlusion frequently develops in arteries that previously appeared to have minimal stenosis or in patients with stable angina. Plaque instability (ruptured plaque with superimposed thrombosis) is a central event in the initiation of acute coronary syndromes [1], associated with intimal inflammation and alteration in hemostatic and coagulation pathways [3]. Several plasma markers of inflammation have been evaluated as potential tools for the prediction of coronary events. Among them are the

markers of systemic inflammation: C reactive protein [4], cytokines (IL-6) [5], adhesion molecules (soluble intercellular adhesion molecule 1 ICAM-1) [6]. All these markers are relevant for the existence of a proinflammatory state, which has an important role in the development of unstable coronary artery disease.

The purpose of our study was to investigate some cellular immune parameters during the development of angina knowing that CHD might involve an inflammatory/autoimmune mechanism [7,8,9,10]. We have investigated the phagocytosis and the superoxide anion release after *in vitro* stimulation of peripheral neutrophils (PMNs), isolated from patients with stable and unstable angina.

Materials and Methods

Materials

The following were purchased from the "Victor Babes" National Institute: zymosan particles (Z), heat aggregated human IgG (aIgG). From Sigma: cytochrome c, Hystopaque, Hank's balanced salt solution (HBSS) supplemented with 2% gelatin, NH_4Cl, eosin.

Patients and control subjects

Patients with stable effort angina and unstable angina were monitored (as in-patients or out-patients) in the Internal Medicine Department of "St. Pantelimon" Emergency Hospital. In the group of unstable angina patients were including according to clinical criteria (rest angina, new onset angina or increasing angina) ECG changes (new, or presumable new, transient ST-segment deviation >/= 0,05 mV or T-wave inversion >/= 0,2 mV in two or more contiguous leads with symptoms) and normal values of cardiac markers (troponins-TnI, TnT-, or CK-MB) [1]. Patients were treated with antiplatelet drugs (aspirin, clopidogrel), anticoagulants (low molecular weight heparins), beta-blockers, and calcium channel blockers. 15 stable and 18 unstable angina patients were investigated.

Healthy volunteers (n=45) (National Hematology Center Bucharest) were investigated concomitantly in order to establish the normal functional parameters of peripheral cellular populations. The donors were of matching sex and ages with the investigated patients and had no antecedents or present cardiovascular diseases symptoms.

Preparation of leukocytes:

Blood was collected by venipuncture in 10 IU heparin x mL^{-1} blood. Within 1-3 h after blood acquisition lymphocytes and neutrophils (PMNs) were isolated by density gradient centrifugation on Hystopaque [11] and red blood cells were lysed with 0.83% NH_4Cl, 0.084% $NaHCO_3$ [12]. Cellular viability, estimated by eosin exclusion test, exceeded 96%.

The in vitro phagocytic activity of human neutrophils

Neutrophils isolated from peripheral blood were incubated for 1h at 37^0C, with inert zymosan particles obtained according to Lachman [13] and investigated by optical microscopy. The results were expressed as the percentage of cells that have engulfed at least one particle.

The respiratory burst of human neutrophils - cytochrome c reduction test.

This test measures superoxide anion release and was performed according to the modified method of Johnston [14] and Olinescu [15]. Cytochrome c is an appropriate detection system for quantitative superoxide anion evaluation. As a consequence of iron reduction after superoxide anion interaction, the cytochrome c spectrum changes; the maximum of absorption of reduced iron rises while the oxidized form falls. The difference between the optical densities (OD550-OD535) x1000 is proportional to the concentration of superoxide anion. Briefly, test samples containing 0.98mg x mL^{-1} cytochrome c, $1x10^6$cells x mL^{-1}, in the presence or absence of various stimuli, adjusted to 1mL with HBSS,

were incubated for 30min at 37°C. We have used zymosan particles for triggering the β-glucane and complement receptor CR3 [16,17] and human heat aggregated IgG mimicking insoluble immune complexes for triggering the Fcγ receptor [18,19]. The optical densities of the test sample supernatants were measured by differential spectrophotometry at 535nm and 550nm using as reference the control sample containing only 0.98mg/mL cytochrome c. The superoxide anion release was estimated as the difference in optical densities [(OD550-OD535) x1000].

Results

The data were presented as mean ± SEM and different groups were compared with the two-tailed t-test - assuming unequal variances **(Table 1)**.

Results and Discussion

Our results obtained on 15 patients with stable angina and 18 patients with unstable angina show that these cardiovascular diseases are characterized by some cellular immune dysfunction that might account for the associated inflammatory reactions reported in literature [20,21,22]. Experimental and clinical data [23] sustain the fact that CHD (especially acute coronary syndromes) have an inflammatory component, which is probably implicated in the pathogenesis of this disease.

Peripheral cell populations isolated from patients with unstable and stable angina

We have observed that the concentration of mononuclear cells isolated from patients with angina are in normal range of values and that in the unstable stage of the angina the number of circulating granulocytes is fairly high compared with normal values (P=0.015). In contrast patients with stable angina present slightly lower concentration of circulating granulocytes, values that confirm the "quiet" stage of the angina **(Figure 1)**. Although the circulating mononuclear cells seem to be in the normal range previous data sustain the fact that these cell population are in an activation stage in unstable angina patients [24,25].

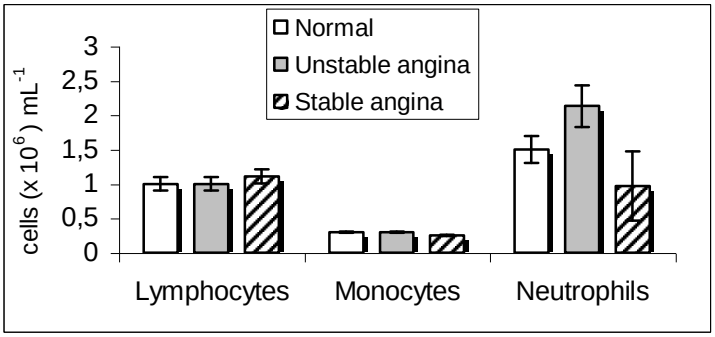

Figure 1. Concentration of cellular population isolated from peripheral blood of normal donors, patients with stable and unstable angina.

The in vitro functionality of peripheral granulocytes isolated from patients with unstable and stable angina

PMNs from atherosclerotic patients adhere to the endothelium and release factors that constrict coronary arteries [26]. It is known that PMNs develop oxygen-dependent cytotoxic mechanisms and the respiratory burst of phagocytes is complex processes in witch reactive oxygen species are vigorously generated [27]. Inhibition of superoxide anion release may have a role in reducing tissue damage, reducing ischemia and reperfusion injury, as superoxide anion is the first oxygen intermediate generated in the respiratory burst of PMNs [28,29]. The respiratory burst of phagocytes is a complex process in which reactive oxygen

species are enzymatically and non-enzymatically generated (ROS) [28,30]: superoxide anion (O_2^{-}), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl). ROS triggers in organism oxidative stress characterized by lipid peroxidation that alters the functional and structural integrity of the biologic membranes, proteins and finally induces genetic direct or indirect modification [30]. All these events contribute to pathogen destruction but can trigger self-destruction also. Control mechanisms, cellular and humoral protect self-structures from the toxicity of ROS: cellular compartmentalization, self-antioxidants and activation of enzymatic systems. The autoprotective factors are localized mainly intracellular, the extracellular medium being highly exposed to the radicalic damage [31]. Phagocytic dysfunction or malfunctions of the respiratory burst regulatory processes are directly or indirectly implicated in various pathology with an inflammatory component [32]. Taking into account the high concentration of circulating granulocytes observed in unstable patients we have investigated their phagocytic capacity and the respiratory burst developed *in vitro*.

Phagocytosis

Phagocytosis is an uptake of large particles governed by the actin-based cytoskeleton. Binding of particles to specific cell surface receptors is the first step of phagocytosis. The receptors able to mediate phagocytosis are expressed almost exclusively in macrophages, neutrophils and monocytes, conferring immunodefence properties to these cells [16]. Emphasizing that peripheral PMNs are nonspecifically activated in cardiovascular diseases [20]. we have studied the *in vitro* capacity to engulf zymosan particles *via* CR3 as a marker for the antimicrobial activity of neutrophils.

The granulocytes isolated from stable angina patients present a higher phagocytic activity **(Figure 2)** compared with unstable angina patients or normal values **(**P=0.002, **Table 1)**. The higher phagocytic activity is not correlated with high concentration of circulating PMNs.

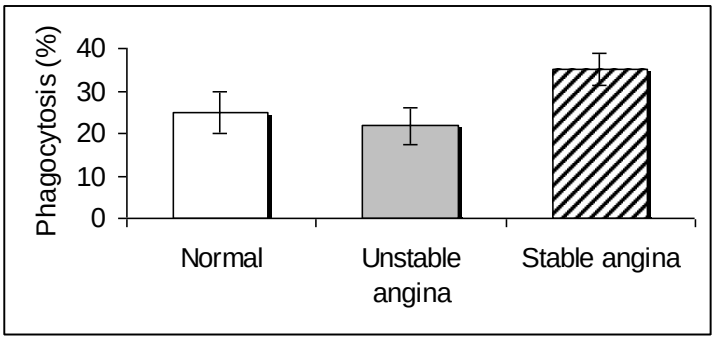

Figure 2. Phagocytic activity of peripheral neutrophils isolated from normal donors, patients with stable and unstable angina.

Oxygen-dependent cytotoxic activity of PMNs isolated from unstable and stable angina patients

At the inflammatory situs PMNs develop oxygen-dependent cytotoxic mechanisms, process that starts with the generation of a toxic oxygen radical, superoxide anion, that enters an enzymatic cascade resulting in further generation of various toxic ROS [28].

Unstimulated superoxide anion release by PMNs isolated from unstable and stable angina patients

PMNs isolated from unstable angina patients generate statistically higher basal superoxide anion compared with stable angina patients or normal subjects (P=0.018) **(Figure 3**, **Table 1)**. High concentrations of peripheral PMNs that exhibit high basal superoxide anion release indicate an active inflammatory reaction in the unstable stage of angina. The

neutrophils isolated from patients with stable angina exhibit normal basal superoxide anion release.

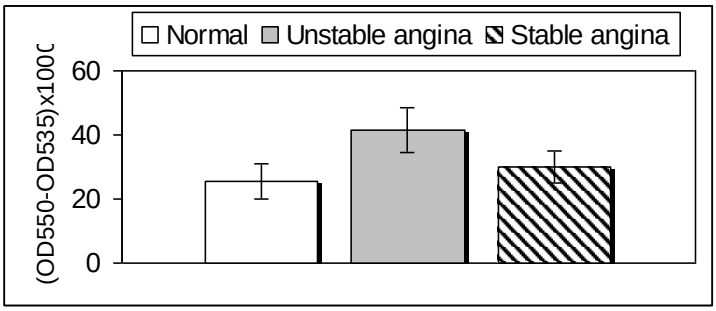

Figure 3. Basal superoxide anion release from peripheral neutrophils isolated from normal donors, patients with stable and unstable angina.

Superoxide anion release by PMNs stimulated via receptor CR3 isolated from unstable and stable angina patients

Although patients with unstable angina present a basal high superoxide anion release the stimulation of PMNs with zymosan that trigger CR3 doesn't induce an abnormal release of this ROS **(Figure 4**, **Table 1)**. The normal phagocytic activity of PMNs isolated from unstable angina patients' correlates with the normal superoxide anion release triggered *via* CR3. It can be postulated that CR3 mediated phagocytosis in unstable angina patients doesn't trigger high levels of superoxide anion release, or that this ROS is dismutated rapidly to other oxygen radicals. Patients with stable angina subscribe to normal values also in the case of CR3 triggered superoxide anion release **(Figure 4**,**Table 1)**, although they have a high phagocytic activity **(Figure 2)**.

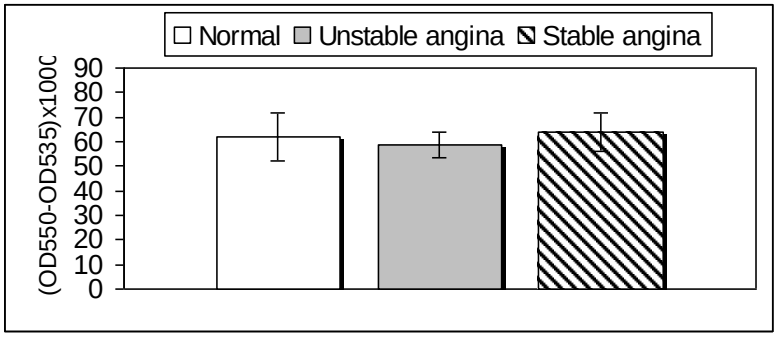

Figure 4. Superoxide anion release after *in vitro* stimulation *via* CR3 of peripheral neutrophils isolated from normal donors, patients with stable and unstable angina.

Superoxide anion release by PMNs stimulated via Fcγ receptor isolated from unstable and stable angina patients

The oxygen-dependent cytotoxic activity of PMNs isolated from unstable angina patients is increased when cells are stimulated *in vitro* with heat aggregated IgG that trigger superoxide anion release *via* FcγR (P=0.009) **(Figure 5**, **Table 1)**. This process is correlated with a high concentration of circulating neutrophils. The patients with stable angina present a normal superoxide anion release triggered *via* FcγR **(Figure 5)**.

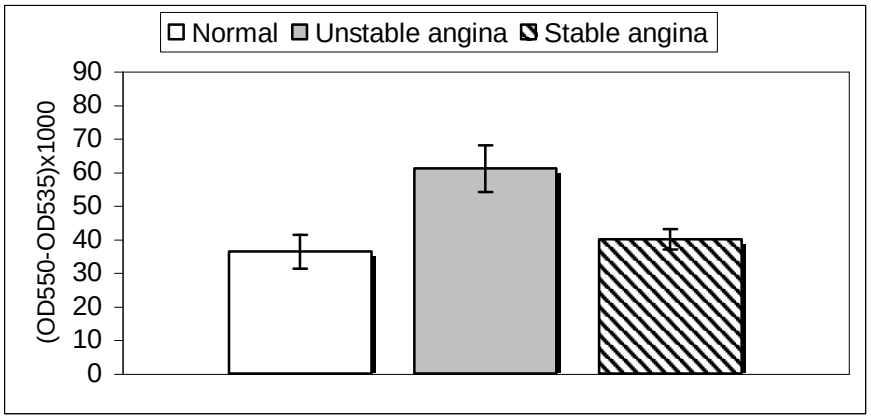

Figure 5. Superoxide anion release after *in vitro* stimulation *via* FcγR of peripheral neutrophils isolated from normal donors, patients with stable and unstable angina.

The enhanced superoxide anion release observed for PMNs isolated from unstable angina patients either unstimulated or stimulated *via* physiological receptors indicate a strong respiratory burst developed implicated in the inflammatory reactions associated with the pathology of CHD disease. Moreover knowing that the generation of NO species is implicated also in the pathology of atherosclerotic vessel [33] and that superoxide anion can also interact with NO generating pathway [34] this observed enhancement of ROS could supply other biochemical pathways that generate toxic reactive species. Our data concerning the activation state of neutrophils are in agreement with previous published data [35,36].

Table 1. Investigated cellular immune parameters compared to normal values, two tail t-test assuming unequal variances

Parameter	**Patients**	
	Unstable angina	**Stable angina**
Peripheral cell populations		
lymphocytes	NS	NS
monocytes	NS	NS
neutrophils	p=0.015	NS
Phagocytosis	NS	p=0.002
Respiratory burst		
-	p=0.018	NS
CR3	NS	NS
FcγR	p=0.009	NS

NS – no significance

Dinamics of the investigated cellular parameters in angina patients

In spite of the fact that overall the cellular parameters of stable angina patients range in normal values we have investigated some stable angina patients during several months and in some cases we have noticed the evolution to unstable angina. The development of an unstable burst was preceded by modification of some immune cellular parameters that indicate an inflammatory outcome of the angina. Therefore the concentration of peripheral neutrophils rises, cells that develop an enhanced basal and FcγR superoxide anion release

(**Figure 6**). The dynamics of CR3 stimulated superoxide anion release and the phagocytic activity of neutrophils doesn't show a modified pattern during the development of the unstable stage of the angina.

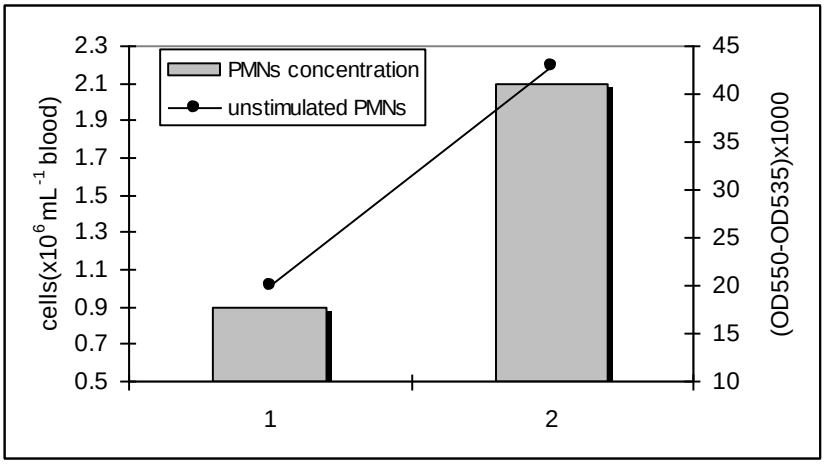

Figure 6. Dynamics of neutrophils peripheral concentration and basal superoxide anion release from a patient investigated in the stable stage of the angina (1) and before developing an unstable stage (2).

Conclusions

In the peripheral blood of angina patients it has been shown that the instability of this disease is due to the lymphocyte activation triggered probably by the monocytes [37]. Moreover monocytes and neutrophils have high expression of adhesion molecules CD11b/CD18 in unstable angina patients [38,39]. A rapid adherence of leukocytes to endothelial cells is one of the first events of the acute inflammatory response in cardiovascular disease. In unstable angina leukocyte activation induce coronary vasoconstriction, thrombocyte activation, thrombotic/thrombolitic processes, all these phenomena leading to the instability of the plaque [39].

The superoxide anion release of unstimulated or FcγR stimulated PMNs isolated from the peripheral blood of unstable angina patients is significantly enhanced compared with normal subject or with the values observed in the stable stage of the angina, function that can contribute to the pathophysiology of the disease [40]. The phagocytic activity of peripheral PMNs is increased compared with normal values in unstable angina, function that is not correlated with a higher superoxide anion release after CR3 stimulation.

Although the clinical symptoms and EKG characteristics of the angina don't point out an unstable stage the immune investigated parameters announce the development of an unstable event.

It can be concluded that the unstable stage of the angina presents alteration of some cellular immune parameters that indicate an inflammatory syndrome associated with CHD disease. We conclude that the immunological parameters are useful to further stratify cardiovascular risk and that therapy should be manipulated accordingly with the immunological status of the patient [41,42].

Acknowledgements

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