Theory of Intercellular Communication in the Development of Endothelial Dysfunction

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 2, pp. 165-170, February, 2012
Original article submitted November 29, 2010

Activation of intracellular signaling and blebbing of the plasma membrane lead to rafting and clustering of membrane receptors. Lymphocyte with high receptor density at the cell pole interacts with endothelial cells, which leads to their hyperactivation. In this case, lymphocyte getting a response from the endothelial cell can release membrane particles, which interact with endothelial receptors and penetrate through gaps between endothelial cells forming aseptic inflammation and causing atherogenesis. Endotheliocytes also contribute to generation of active membrane microparticles. Hyperactivation of endothelial cells and constant stimulation by the lymphocytes and microparticles trigger programmed cell death resulting in exfoliation of the endothelial cell. The endothelial defect is replaced by endothelial cells of the vascular wall (in case of mild endothelial dysfunction) or by progenitor endothelial cells (in case of severe dysfunction).

Key Words: *endothelial dysfunction; clustering; blebbing; microparticles*

Vascular endothelium as the tissue of a multicellular organism depends on not only physical and chemical conditions of the external (atmospheric pressure, blood pressure, applications of outside influence, *e.g.,* radiation or compression, foreign objects, *etc*.) and internal environment (bioactive substances, hormones, neurotransmitters, toxins, allergens, *etc*.), but also on direct cell-cell contacts and metabolites, including cytokines, signaling molecules, *etc*. [1].

Endothelial dysfunction (ED) is a polygenic, multifactorial disease defined as altered morphological and functional state of endothelium accompanied by the production of biologically active substances (vasoconstrictors or vasodilators, chemoattractants, cell adhesion factors, platelet aggregants or anti-aggregants, *etc*) in amounts inadequate to the needs of the organism. This condition occurs due to hyperactivation of endothelial cells and leads to their apoptosis, "endothelial wound", aseptic inflammation of the vascular wall and parietal thrombosis, and atherogenesis, which results in multilevel vascular pathology and closes the vicious circle of progressive dysfunction of endothelial cells.

Intercellular contacts via microtubules, desmosomes, receptor-ligand interactions or antigen/antibody, *etc*., play the major role in the regulation of functional activity either of the endotheliocytes and vascular and blood cells, as well as tissues nourishing the vessels. Peripheral blood lymphocytes are highly sensitive cells with immunological memory and a large spectrum of regulatory effects on vascular and other cells through direct cell-cell contacts and formation of microparticles, cytokines, and bioactive substances [3].

According to recent concepts, an immune synapse is formed between T lymphocyte and antigen-presenting cell associated with altered physicochemical properties of the membrane.The participation of blebbing (membrane protrusions) in the formation of immunological synapse is confirmed by the presence of antigens in the blebbs [10].

From the viewpoint of the formation of immune synapses, it becomes evident that lymphocytes are

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0007-4888/12/1532-0201

involved into the pathological process in vascular diseases related to target organ damage. This is due to the fact that the lymphocyte is a memory cell, capable of transferring information from the pathological focus to the regulatory structures (in particular, other blood or vascular cells). Due to the production of cytokines and humoral factors, reprogramming of structures interacting with lymphocyte occurs in order to change the homeostasis adequately in conditions of evolved acute or chronic hypoxia [4,10].

There are lipid domains (rafts) in the plasma membrane, which are more densely packed dynamic lipid associates. Membrane proteins involved in cell contact are located on lipid rafts. The interaction of receptor with ligand results in increased mobility of these receptors in the membrane lipid bilayer and clustering that potentates signal perception in the cell. Clustering of receptors can lead to desquamation of membrane microparticles from membrane surface bearing receptors and their clusters [13].

Thus, blebbing of blood and/or endothelial cells with subsequent formation of membrane-derived microparticles plays a key role in the genesis of ED. Membrane-derived microparticles in biological fluids formed by cell activation or apoptosis produce multiple functional biological effects involved in modulation of physiological functions such as immunity, inflammation, angiogenesis, hemostasis, and thrombosis [3,9].

The aim of the study was to determine the influence of lymphocytes on the generation of endothelial microparticles *in vivo*.

MATERIALS AND METHODS

The study involved 359 male and female patients at the age of 30-65 years suffering from hypertension (n=80), hypertension and coronary heart disease (n=94), hypertension and acute stroke (n=110), refractory hypertension (n=15), neurocirculatory dystonia (n=20), as well as healthy individuals with no history of cardiovascular diseases (n=40). All participants gave informed consent to participation in this study.

Peripheral venous blood (5 ml) was collected into tubes containing 0.15 ml heparin. Lymphocyte fraction was isolated by centrifugation on Ficoll-verografin density gradient (ρ=1.077) at room temperature for 15 min at 3000 rpm. The isolated cells were centrifuged for 5 min at 3000 rpm. PBS (phosphate buffered saline) or Hank's solution (500 μl) were then added to the precipitate. Microscopic examination was performed in 10 fields of view (viewing area 4 cm^2) using phase-contrast attachment (×900). Lymphocytes were seen as round cells with a diameter of 9-10 μ. The following types of lymphocytes were identified by plasma membrane morphology: intact cells with visually unchanged plasma membrane, round and smooth surface; cells in a state of blebbing with protrusions of the plasma membrane.

In parallel, membrane microparticles were isolated from the peripheral blood by ultracentrifugation (Rcf 11,000, 5°C, 2 min). The obtained supernatant was centrifuged (Rcf 13,000, 5°C, 45 min.) Primary antibodies to endothelial cell antigen CD62E or lymphocyte antigen CD38, the main blood lymphocyte antigen (Sigma-Aldrich), were added (50 μl, diluted 1:100 in PBS with 1% fetal goat serum), incubated at 37°C for 1 h, and washed in PBS according to standard protocol. Secondary-PI-labeled antibodies (10 μl; Sigma-Aldrich) were added to the obtained sediment, incubated at 37°C for 30 min, washed in PBS, and resuspended; after that 20 μl suspension was applied onto a slide (viewing area 4 cm^2) and analyzed under a fluorescence microscope (x900) in 10 fields of view.

The severity of ED was determined by the number of desquamated endothelial cells by the method [13].

Statistical analysis was performed using the Kolmogorov–Smirnov test, Mann–Whitney U test, Wilcoxon test, and ANOVA.

RESULTS

The content of circulating endothelial microparticles increases with increasing the number of lymphocytes in the state of blebbing by 40% (r=0.81, p=0.0033); the number of circulating lymphocyte microparticles also increased, but the correlation was insignificant (r=0.56).

The degree of lymphocytes involvement into intercellular communication varied during ED development in different patients with the same pathology. We have noted that high involvement of lymphocytes into ED and generation of endothelial membrane particles tends to dominate in patients with acute stroke and progression of coronary artery disease (in 93.5±3.5% cases, p<0.001). In patients with essential hypertension, normal content of lymphocytes (100 cells per 4 cm^2) with a low number of lymphocytes in the state of blebbing (76.9±2.8 cases, p=0.014) was recorded against the backdrop of increased generation of endothelial microparticles indicating low involvement of lymphocytes in ED. In refractory hypertension, enhanced number of lymphocytes (257.6±3.8 cells per 4 cm^2) in the blood (100%, p=0.052) against the background of increased content of endothelial microparticles (257.2±1.3 per 4 cm^2) and a low number of lymphocytes in the state of blebbing were documented. Generation of membrane microparticles by lymphocytes (179.0±2.2 per 4 cm^2) correlated with increase in endothelial and lymphocyte microparticles (r=0.91, p=0.89). This fact explains the resistance to

drugs, because lymphocytic and endothelial microparticles can bind cell receptors. With neurocirculatory dystonia, regarding higher content either of endothelial microparticles (150.6±4.1), low content of lymphocytes in the state of blebbing (13.0±2.7%), and total number of lymphocytes with and without protrusions of plasma membrane (69.7±4.4 cells per 4 cm^2), we suppose that the development of endothelial dysfunc-

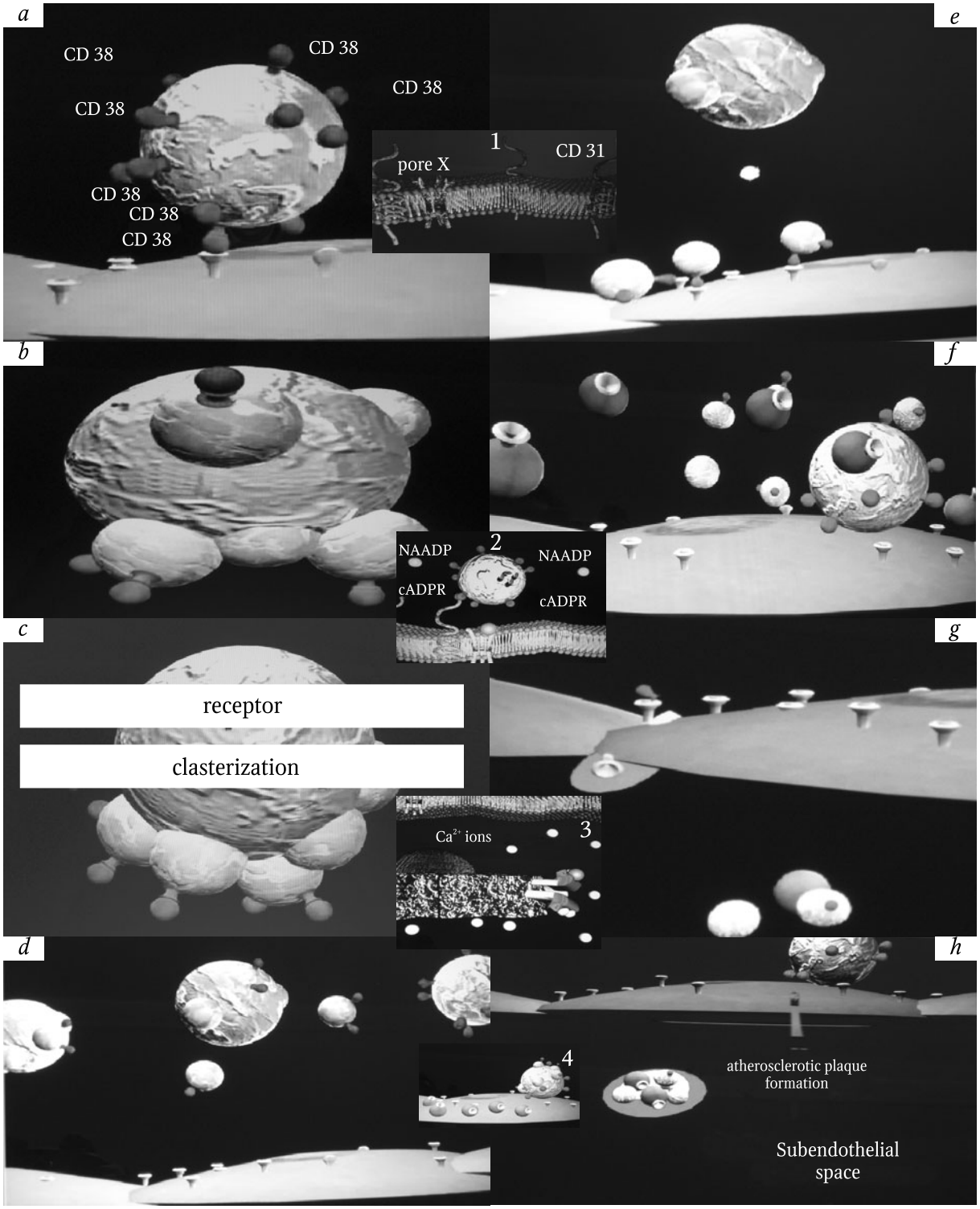

Fig. 1. Theory of intercellular communication during ED development as exemplified by lymphocyte-endothelial interactions mediated by clustering of the receptor apparatus (*a-c*) and release of membrane-derived microparticles (*d-h*). Insets 1-4 present one of the sequences of events.

tion is largely due to factors other than immune synapses with lymphocytes. In controls, low amounts of endothelial microparticles (15.4±4.7) and lymphocytes in the state of blebbing (an average of 5.2±3.3%) were recorded. However, in some patients the percentage of lymphocytes in the state of blebbing was about 20% at low levels of endothelial microparticles, indicating cell-cell contacts which were insignificant for the development of ED. Lymphocyte blebbing can be explained by other effects, which requires widening of the diagnostic search for these patients.

According to our study, blood levels of circulating membrane microparticles increased several times with severe ED accompanying the critical condition of the cardiovascular system [9,13]. The more pronounced the degree of vascular damage in cardiovascular diseases, the higher the percentage of lymphocytes in the state of blebbing. This indicates the process of clustering of the receptor system [3,10]. In this context, elevated levels of endothelial membrane-derived microparticles in the blood become understandable. Correlation coefficient 0.81 (p=0.0033) between the percent of blebbing lymphocytes and the level of endothelial particles confirms the advantage of lymphocyte/endotheliocyte response, that, in particular, leads to triggering of the intracellular cascade systems, and changes in membrane-cytoskeleton interactions in endotheliocyte [12].

Significant increase in phospho p38 expression in endothelial cells occurs during ED (data not presented). p38 belongs to mitogen-activated protein kinases sensitive to the effects of stress, growth factors, and inflammation. Increased production of p38 is associated with the signaling cascade resulting in programmed cell death [7]. Interestingly, at the same time membrane-derived microparticles contain increased amounts of JNK which prevails over p38 and, to a greater extent, over ERK. In other words, under conditions resulting in ED, endothelial cells synthesize more proteins that promote apoptosis rather than preserve cell viability [1,15]. It is also known that the activation of JNK and p38 synthesis is associated with an increased concentration of intracellular calcium and or free radicals in the intracellular space. Triggering of the pathological pathway mediated by mitogen-activated protein kinases lead and the presence of increased intracellular calcium levels, uncoupling of cytoskeletal proteins, and externalization of phosphatidylserine, and rafting (displacement of lipid domains) (Fig. 1, *a*, *b*, 1, 2, 3). Lipid rafts enriched in cholesterol and phospholipids are a highly mobile structure capable of moving on the surface of phospholipids [11]. Moreover, incorporation of membrane proteins into the raft makes it more stable; receptor/ ligand interactions of embedded protein triggers cell signaling. As we have shown previously [3], rafting and dynamic movement of cell membranes contribute to the convergence of receptor loci predominantly of the same type to one cell pole; membrane receptors cluster occurs (Fig. 1, *c*), characterized by high cross-reactivity, resistance and enhanced unidirectional formation of the signal that potentiates the implementation of the effect. Microscopically, cluster is manifested by accumulation of blebs (protrusions) on one cell pole which enables the study of rafting and clustering. Under physiological conditions corresponding to non-activated state of endothelium, cells of endothelial lining have chemical properties of the natural repulsion of blood cells that promotes preserving of blood rheological characteristics [1]. Under physiological or pathological ED, endothelial cells primarily interact with immune cells [2,12,14]. So, the lymphocyte with increased density of the receptor apparatus at the cell pole interacts with endothelial cells causing their hyperactivation. Getting a response from the endothelial cell, lymphocyte can release membrane particles (0.1-1.0 μ), which are vesicles with biologically active substances surrounded by lymphocyte membrane and bearing its receptors (Fig. 1, *d*). In turn, microparticles [6,8] can interact with receptors of endothelial cells and significantly block them (Fig. 1, *e*). The number of circulating membrane microparticles is many times greater than the number of lymphocytes, and their ability to transfer cell-membrane receptors of parent cell determines their high effector capacity [10]. Membrane microparticles penetrate through gaps between endothelial cells [14] forming aseptic inflammation and causing atherogenesis (Fig. 1, *g*, *h*). Hyperactivated endothelial cells are subjected to the same changes as the membrane of lymphocytes. Endothelyocytes involve in the generation of active membrane microparticles, which may come into contact with the same or other endothelial

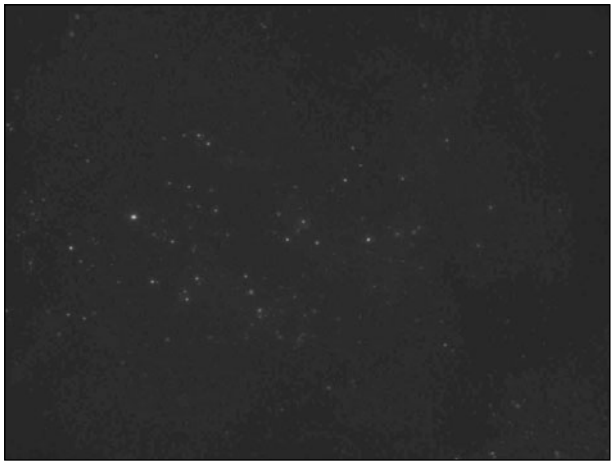

Fig. 2. Endothelial PI-labeled microparticles CD62E$^+$. Luminescent microscopy, ×900.

cells, and blood cells such as lymphocytes, activating them and providing the antigenic information about endotheliocyte (Fig. 1, *f*, *g, h*, 4). Hyperactivation of endothelial cells and constant stimulation by lymphocytes and microparticles trigger programmed cell death resulting in the exfoliation of the endothelial cell. In mild ED, endothelial wound is replaced by endothelial cells of the vascular wall; with severe ED, by endothelial progenitor cells. [5]. Clinically, ED is associated with the deposition of calcium salts and impaired elasticity of the vascular wall.

Blockade of cells receptors with circulating membrane microparticles or change in the intracellular cascade systems with respect to the drug should be considered as one the possible negative effect of microparticles (Fig. 2). Thus, refractory hypertension shows elevated overall level of lymphocytes and blebbing lymphocytes as well as increased number of circulating endothelial and lymphocyte microparticles correlating with coefficient 0.91 (p=0.89). This effect is quite surprising as we have previously shown significantly reduced circulating membrane microparticles in the blood of patients with essential hypertension under the influence of antihypertensive therapy [3]. In the case of refractory hypertension, lack of the desired effects of the drug is confirmed by high content of membrane microparticles circulating in the blood.

Thus, lymphocytes are involved in pathogenesis of ED at various cardiovascular diseases by enhancing receptor system or generation of antigen-bearing membrane microparticles.

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