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Cellular mechanisms of human atherosclerosis: Role of cell-to-cell communications in subendothelial cell functions

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ABSTRACT

The present study was undertaken in order to extend of our earlier work, focusing on the analysis of roles of cell-to-cell communications in the regulation of the subendothelial cell function. In present study, we have found that the expression of connexin43 (Cx43) is dramatically reduced in human atherosclerotic lesions, compared with undiseased intima. In atherosclerotic lesions, the number of so-called 'connexin plaques' was found to be lower in lipid-laden cells than in cells which were free from lipid inclusions. In primary cell culture, subendothelial intimal cells tended to create multicellular structures in the form of clusters. Cluster creation was accompanied by the formation of gap junctions between cells; the degree of gap junctional communication correlated with the density of cells in culture. We found that atherosclerosis-related processes such as DNA synthesis, protein synthesis and accumulation of intracellular cholesterol correlated with the degree of cell-to-cell communication. The relation of DNA and protein synthesis with cell-to-cell communication could be described as "bell-shaped". We further incubated cells, cultured from undiseased subendothelial intima, with various forms of modified LDL causing intracellular cholesterol accumulation. After the incubation of intimal cells with modified LDL, intercellular communication has "dropped" considerably. The findings indicate that intracellular lipid accumulation might be a reason for a decrease of the number of gap junctions. The findings also suggest that the disintegration of cellular network is associated with foam cell formation, the process known as a key event of atherogenesis.

1. Introduction

The organization of the human arterial intima has been intensely studied (Virchow, 1856; Langhans, 1866; Maximow, 1927; Anichkov, 1947; Haust, 1986; Stary, 1989; Schwartz et al., 1995; Bobryshev and Lord, 1996; Orekhov et al., 2010) but it is not yet well understood (Orekhov et al., 2014). It has been demonstrated earlier that in the subendothelial intima of adult human aorta the majority of cells are not separated from each other but form a three-dimensional cellular network within which cells contact each other by means of their cell processes (Rekhter et al., 1991). This three-dimensional cellular network consists mainly of smooth muscle cells and pericyte-like stellate cells (Bobryshev and Lord, 1996; Andreeva et al., 1998; Orekhov et al., 2014). The network is formed by gap junctions located on long processes of intimal cells (Andreeva et al., 1995; Bobryshev and Lord, 1996), paving the subendothelial bed of all vessels from the very small vessels and extending to the largest arteries and veins (Andreeva et al., 1995). The cellular network is found to be partly disintegrated in early atherosclerotic lesions (Rekhter et al., 1991). In fatty streaks and atherosclerotic plaques many cells lose all contacts (Rekhter et al., 1991; Andreeva et al., 1997). In advanced atherosclerotic plaque, cells have lost all contacts with each other (Andreeva et al., 1997).

The integration of alpha-smooth muscle actin expressing cells, such as smooth muscle cells and pericyte-like stellate cells, in the form of a common cellular network is necessary for the normal physiology of the tissue while disintegration of this network might play a significant role in atherosclerosis (Andreeva et al.,

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1997). It is well appreciated that gap junctions represent electron-microscopically identifiable morphologically distinctive structures, essential for the organization of cell-to cell contacts (Nielsen et al., 2012). Gap junctions in all organs have a uniform structural appearance; connexins, in particular connexin 43(Cx43), are well known to represent specific molecules involved in the formation of gap junctions (Nielsen et al., 2012; Iyyathurai et al., 2013; Aasen, 2015; Defamie et al., 2014; Oshima, 2014; Solan and Lampe, 2014; Zhou and Jiang, 2014).

The present study was undertaken in order to extend of our earlier work (Andreeva et al., 1995, 1997, 1998; Bobryshev and Lord, 1996; Orekhov et al., 2010, 2014; Bobryshev et al., 2011; Ivanova et al., 2015), focusing on the analysis of roles of cell-to-cell communications in the regulation of the subendothelial cell function and intimal homeostasis. The present study has revealed that alterations of cell-to-cell contacts between cultured subendothelial intimal cells lead to dramatic changes in cellular functions relevant to atherosclerosis. In this study, we also found that foam cell formation in culture, induced by modified low density lipoprotein, may be the cause of a "weakening" and the disappearance of contacts between subendothelial intimal cells. The findings suggest that the disintegration of cellular network is associated with foam cell formation, the process known as a key event of atherogenesis.

2. Materials and methods

2.1. Materials

Medium l99, DMEM, fetal calf serum (FCS), Fungizone, Kanamycin, Glutamine, trypsin were purchased from Gibco BRL (Paisley, UK). Disposable tissue culture materials were obtained from Nunc (Roskilde, Denmark), and cover glasses from Polysciences Europe GmbH (Eppelheim, Germany). Collagenase type IV, Oil Red O, calcein, monoclonal anti-alpha smooth muscle actin antibodies were purchased from Sigma Chemical Co. (St. Louis, USA).

2.2. Procurement of aortic tissue

Human aortas were obtained at urgent autopsy from 32 to 58-year old men 3–4 h after sudden death. The study was carried out in accordance with the principles outlined in the Helsinki Declaration of 1975, as revised in 1983 and was approved by the Ethics committees of the Institute of General Pathology and Pathophysiology, Russian Academy of Sciences, and by the Institute for Atherosclerosis Research, Skolkovo Innovative Center, Moscow. The vessels were opened lengthwise under aseptic conditions, the adventitia was removed mechanically. Intima-media was rinsed with phosphate buffered saline (PBS), pH 7.6.

The arteries were opened longitudinally and again washed with PBS. The grossly normal parts of the arteries and intimal parts with atherosclerotic lesions were identified macroscopically and classified according to the guidelines of Atherosclerosis Council of the American Heart Association (Stary et al., 1994, 1995). Undiseased areas of the intima, called also as diffuse intimal thickening (or lesion type 0) (Stary et al., 1994), were characterized by a smooth luminal surface. Intimal zones with initial atherosclerotic changes (lesion type I) corresponded to parts of arteries with a smooth yellowish surface with occasional small yellow spots. According to routine histology, small aggregates of extracellular lipid droplets were present in the connective tissue matrix. No other changes in the tissue structure were detected. Macroscopically, fatty streaks (lesion type II) were yellow strips and spots that slightly protruded over the vessel surface. Fatty streaks often merged into larger structures, forming lesion clusters. In tissue sections, the presence of lipids was identified inside cells. The connective tissue matrix also contained extracellular lipids. Lipofibrous plaques (lesion type Va) were spherical or elliptic protrusions of yellowish or nacreous color. Changes characteristic of fatty streaks were defined microscopically. They included accumulated intracellular lipids and increased content of extracellular matrix. Lipofibrous plaques contained a bulky necrotic core covered by a connective tissue layer and also included zones morphologically resembling fatty streaks. Fibrous plaques (lesion type Vc) were considerably protruding, rounded or oval, and pearl-colored formations. They were mostly composed of a crude connective tissue matrix with embedded cells. Samples from grossly normal (diffuse intimal thickening) and atherosclerotic sites (initial lesion zones, fatty streaks, lipofibrous and fibrous plaques) were excised across the longitudinal axis of the vessels.

2.3. Primary culture of human aortic intimal cells

Grossly normal vascular segments, clusters of fatty streaks and atherosclerotic plaques were cut off under sterile conditions. The intima was mechanically separated from the media along the internal elastic lamina. Tissue samples were dispersed by 0.1% collagenase for 3 h at 37 °C. After washing, cells were seeded into 35 mm plastic culture dishes on cover glasses ($1–2 \times 10^5$ cells per dish). The cells then were cultured in Medium 199 containing 10% FCS, 2 mM Glutamine, 100 mg/ml Kanamycin, 2.5 mg/ml Fungizone, as described elsewhere (Orekhov et al., 1986a).

On the sixth-seventh day, cell types of cultures were identified by immunocytochemistry as used previously (Andreeva et al., 1992). The cellular composition of primary cultures, derived from undiseased intima and atherosclerotic lesions, is shown in Table 1. As seen from Table 1, the major cell type is represented by cells which contained alpha-smooth muscle actin (90%). In addition to typical smooth muscle cells, alpha-smooth muscle actin is associated with pericyte-like cells stained also with antibodies 3G5 and 2A7 (Andreeva et al., 1998; Orekhov et al., 2014). Inflammatory cells detected by antibodies against CD45 and CD68 represent a minority of the total cell population. In cultures, derived from atherosclerotic plaque, a proportion of inflammatory cells were found to be significantly higher but the majority of cells was represented by alpha-smooth muscle actin positive cells (80%) (likewise as in cell culture of undiseased intima). Apparently, the endothelium was completely lost during washing of aortic tissue specimens as endothelial cells were not found in cultures from undiseased areas and in cultures from atherosclerotic lesions.

2.4. Measurement of intercellular communication by fluorescent dye transfer

On the sixth or seventh day in culture, the cells were washed with Medium 199 containing 10% FCS. Parameters of intercellular communication were determined in cell cultures after microinjection of a 95 mM calcein solution (in distilled water) in a single cell. The dye was microinjected in a microelectrophoresis system with standard glass microelectrode, resistance 70–100 MΩ and pulse injection for 5 s. The percentages of dye-coupled cells were determined as follows. Ten min after dye injection, cell cultures were examined using a Photomicroscope III (Opton Feintechnik GmbH, Oberkochen, Germany) at low magnification (eyepieces 10, objective 16). The total cell number per view field square (0.8 mm^2) was determined using phase-contrast microscopy. The incidence of dye coupled cells in the same field was scored under epifluorescence system optimized for FlTC-fluorescence (BP 450–490, LP 520). Closely opposed fluorescent cells were distinguished for counting with phase-contrast microscopy of the same cells at higher magnification. Three coverslips containing at least 20 injected cells were studied in each cell culture.