Proliferation Characteristics of Cultured Human Aortic Endothelial Cells and Expression of Adhesion Molecules

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 122, No. 8, pp. 214-217, August, 1996
Original article submitted April 28, 1995

Basal expression of the adhesion molecules P-selectin and ICAM-1, which mediate adhesion and transendothelial migration of leukocytes, by endothelial cells of human aorta and umbilical vein and its relationship with the proliferative behavior of these cells are studied in primary prolonged cultures. Inverse proportionality between the percentage of resting endothelial cells and those expressing the adhesion molecules in preconfluent and confluent monolayers, on the one hand, and the percentage of cells in the active cycle which do not express the adhesion molecules, on the other, is demonstrated. This relationship is one of the major causes of the *in vitro* functional heterogeneity of the endotheliocyte population in the expression of adhesion molecules and its adhesiveness for leukocytes.

Key Words: *human endothelial cells; adhesion molecules; in vitro proliferation*

The endothelium of adult human aorta is represented by a heterogeneous population of cells differing in size, number of nuclei, ploidy, *in vivo* protein content [15], proliferative behavior [5], expression of adhesion molecules, and adhesiveness for blood cells [9,13] *in vitro*. The causes and mechanism of such a heterogeneity in the population of endothelial cells (EC) in human ontogenesis are unclear [5,15].

After the contribution of the endothelium to the development of atherosclerosis had been established [14], possible links between endothelial heterogeneity and local manifestations of atherosclerotic lesions of the aortic intima were investigated [5,13,15]. Since peripheral blood leukocytes migrating into the intima directly contribute to early atherogenesis [14], it seems reasonable to study the interactions between EC of different phenotypes and leukocytes during transendothelial migration. For this purpose a culture of aortic endothelium can be used. In a confluent state it forms a morphologically heterogeneous monolayer similar to endothelial lining of the aorta [5,15]. Part of giant EC in such cultures differ from the smaller ones by higher adhesiveness for neutrophils and monocytes and by higher basal expression of P-selectin and ICAM-1 (intercellular adhesion molecule-1) [13], which are involved in leukocyte migration into the intima at the early stages of atherogenesis [8,11,14].

In order to elucidate the cause of functional heterogeneity of aortic EC, we investigated basal expression of P-selectin and ICAM-1 by phenotypically different EC with different proliferative behavior in the culture. For comparison, experiments were performed on human umbilical vein endothelium (morphologically homogenous EC population [1]).

MATERIALS AND METHODS

Primary cultures of aortic and umbilical vein endothelium were obtained as described previously [1,5]. For monolayer cultures EC were seeded at a density of 3 to 5×10^4 cells/cm^2 and for discrete colony clones EC were plated at a density of 5 to 10

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0007-4888/96/0008-0842$15.00 ©1997 Plenum Publishing Corporation

cells/cm². The scheme of radioautographic and immunocytochemical analysis of proliferative behavior of EC growing in a confluent culture is shown in Fig. 1. Endothelial cells were labeled with ^{3}H-thymidine (1 μCi/ml medium) 30 min before fixation (Fig. 1, *a*). For the determination of proliferating pool labeled DNA precursor (^{3}H-thymidine) was added when the medium was replaced at least 48 h before fixation (Fig. 1, *b*). The preparations were coated with nuclear photoemulsion (type M, KHIM-FOTO Research Institute) and after a 3-day exposure were routinely processed [3]. The pool of proliferating EC can be determined by immunocytochemical detection of proliferating cell nuclear antigen (PCNA) [10]. The expression of adhesion molecules was detected with monoclonal antibodies (MAb) S12 to P-selectin [8] provided by Dr. R. P. MacIver (University of Oklahoma, USA) and 10F3 MAb to ICAM-1 provided by Dr. O. Yu. Printseva (Cardiology Research Center, Russian Academy of Medical Sciences). The expression of PCNA was detected with MAb PC10 (DACO). Different localization of the antigens (PCNA in the nucleus, ICAM-1 on the cell surface, and P-selectin in the cytoplasm) allowed us to detect them in pairs: PCNA—ICAM-1 and PCNA—P-selectin. Bound antibodies were detected using standard avidin-biotin-peroxidase or peroxidase-antiperoxidase (PAP) methods [4] in all experiments. The intensity of staining of the cytoplasm and nuclei was assessed visually (in 1000 EC for each preparation). The amounts of P-selectin- or ICAM-1-positive cells among EC in the active cycle and among resting EC were compared both in growing cultures and in cultures which had attained confluence as well as in prolonged cultures of umbilical vein and aortic EC (passages 0-3, 10, 15 and 0-8, 15, 20, and 25, respectively).

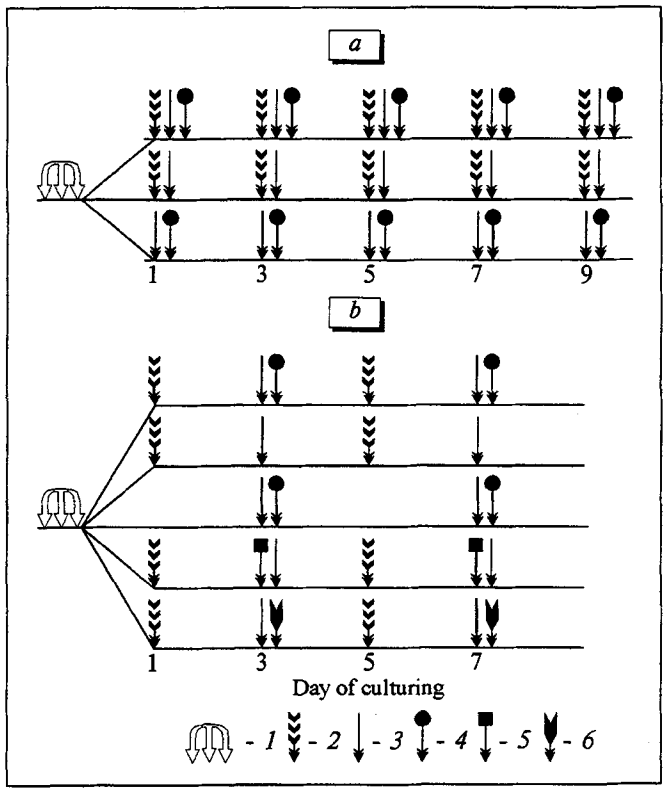

Fig. 1. Scheme of experiments on expression of P-selectin and ICAM-1 in growing and confluent cultures of human aortic and umbilical vein EC with different proliferative behavior. Experiments with pulsed (*a*) and saturated (*b*) labeling of EC with ^{3}H-thymidine (assessment of the proliferative pool of EC). *1*) seeding of EC in a culture; *2*) addition of ^{3}H-thymidine; *3*) fixation; *4-6*) immunocytochemical staining with MAb recognizing PCNA, ICAM-1, and P-selectin, respectively.

RESULTS

Immunocytochemical study of the distribution of adhesion molecules in both populations of EC during prolonged subculturing revealed a similar dynamics of changes in the expression of ICAM-1 and P-selectin. A decrease in the share of EC expressing these molecules was seen in primary cultures attaining confluence. During subculturing, a progressive decrease in the share of antigen-positive cells was observed up to passages 8-10, the decrease in the share of P-selectin-positive EC being more rapid. At later passages, the share of EC intensively expressing the adhesion molecules markedly increased again, approaching the initial level and even surpassing it (Fig. 2). It was hypothesized that changes in the expression of adhesion molecules are due to the characteristic changes in the proliferative behavior of EC during subculturing [7]. To test this hypothesis, we compared the expression of adhesion molecules in proliferating and resting aortic and umbilical vein EC during several passages.

In addition to the bulk of small diploid cells, the *in vitro* proliferating pool of aortic EC contains considerable amounts of polyploid mono- and binuclear EC (data on the ploidy are not shown). In this subpopulation, the expression of ICAM-1 and P-selectin by the majority of EC during culturing was weak or absent. At the same time, nonproliferating cells, predominantly giant mono- and polynuclear EC, expressed the adhesion molecules with different intensity (up to very high) even after multiple passages. It is noteworthy that during subculturing of initially morphologically homogenous umbilical vein endothelium [1] phenotypical variety of EC markedly increased: giant mono- and binuclear EC of high ploidy appeared *de novo* among small diploid cells with a very low expression of P-selectin and ICAM-1 [13]; some of them do not proliferate and intensively expressed the adhesion molecules (Fig. 3). By mor-

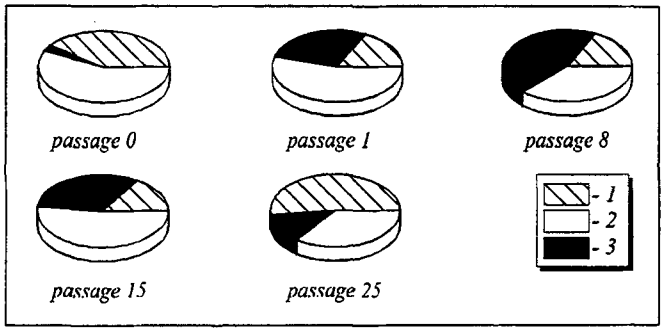

Fig. 2. Expression of ICAM-1 by human umbilical vein EC in confluent culture during prolonged subculturing. Changed in the share of PAP-positive and PAP-negative cells. PAP reaction: *1*) highly positive; *2*) positive; *3*) negative.

phology and type of expression of the adhesion molecules this monolayer was similar to the aortic endothelium culture after first passages. Inverse proportionality between the expression of each of the adhesion molecules and the expression of PCNA or incorporation ^{3}H-thymidine (saturating label) (Fig. 3) was preserved until confluence: the 15th passage for aortic EC and the 25th passage and for umbilical vein EC.

In order to elucidate histogenetic mechanism underlying the increase in the heterogeneity of endothelial monolayer during culturing, we examined in discrete clones phenotypical changes in the "offsprings" of separately cultured umbilical vein and aortic EC during their "replicative aging" [6]. The PCNA(+), ICAM-1(-) phenotype predominated among replicatively "younger" (generations 1-7) small

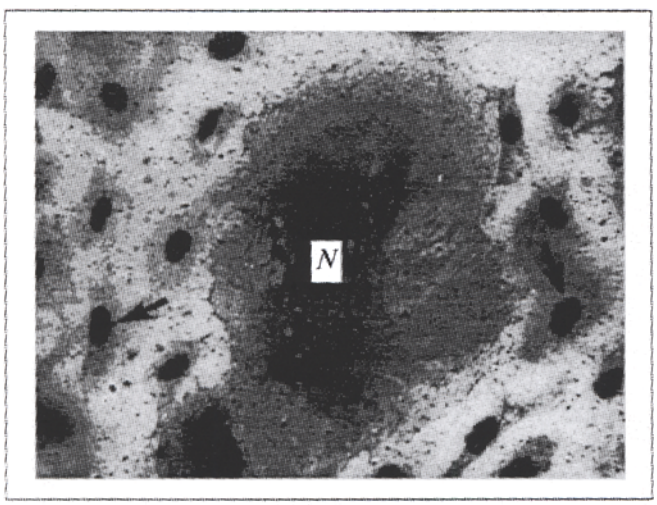

Fig. 3. Expression of ICAM-1 and ^{3}H-thymidine incorporation in confluent monolayer of umbilical vein EC, passage 13. Endothelial cells were cultured with ^{3}H-thymidine for 72 hours (days 3-5 in culture). The culture was stained by the avidin-biotin-peroxidase method [4] using MAb 10F3. The nuclei were stained with hematoxylin, ×270. Small (diploid) ICAM-1-negative cells contain an autograph of ^{3}H-thymidine in the nuclei (shown by arrows). Giant (polyploid) ICAM-1-positive cell contains no autograph in the nucleus. *N*: nucleus.

diploid cells which were located in the center of colonies and appeared as a result of the most active proliferation. Replicatively "older" EC (generations 8-12) at the periphery of colonies had a higher ploidy. This population contained EC with ICAM-1(-), PCNA(+) phenotype (some of them incorporated ^{3}H-thymidine, which indicates DNA replication [6], and did not express the adhesion molecules) and EC with ICAM-1(+), PCNA(-) phenotype, which did not incorporate ^{3}H-thymidine (pulse label) and probably were in active proliferative cycle, resuming the expression of adhesion molecules.

Thus, it was shown that active proliferation of EC is associated with a decrease in the expression of adhesion molecules. However, resting EC, mainly giant polyploid mono- and polynuclear, accumulating during replicative aging in prolonged cultures retain a very high level of expression (Figs. 2 and 3).

These findings provided more insight into the causes of EC heterogeneity *in vitro*. The different degree of "replicative aging" and polyploidization of EC attained in the course of ontogenesis of the endothelium *in vivo* is the major cause. It determines the differences in the proliferative behavior of EC of different phenotypes in culture: small cells capable of active proliferation and quiescent giant cells. Another cause is the position of EC in the monolayer, i.e., the possibility to spread on the substrate is one of the most important limiting factors for entering the proliferative cycle [2,12]. That is why EC with a similar proliferative potential have different possibilities to realize their replicative program. Different proliferative behavior is associated with the reprogramming of intracellular synthesis aimed at fulfilling some specific functions [2,6], which results in a higher phenotypical and functional heterogeneity of EC *in vitro*.

The study was supported by the Russian Foundation for Basic Research (project code 94-04-21864).

We are grateful to Dr. R. V. Latsis for his help in the discussion of results.

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Micro- and Ultrastructure of the Pial Arteries in Experimental Hypercholesterolemia

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 122, No. 8, pp. 218-221, August, 1996
Original article submitted June 20, 1995

Morphological changes induced by experimental cholesterolemia in the walls of pial arteries of Chinchilla rabbits are studied by light and electron microscopy. It is found that these changes are typical of small artery atherosclerosis.

Key Words: *hypercholesterolemia; atherosclerosis; pial arteries*

Atherosclerosis is the major cause of chronic cerebral circulation disorders [5,7]. Information regarding the atherosclerosis-related morphology of cerebral and pial blood vessels is scarce [1,3,11]. Our objective was to examine morphological changes occurring in pial arteries in experimental hypercholesterolemia (HC).

MATERIALS AND METHODS

Hypercholesterolemia was routinely induced in 3-year-old male Chinchilla rabbits weighing 3 to 3.5 kg by daily injections of 10% oil suspension of cholesterol in a dose of 0.2 g/kg for 3 months [9]. Group 1 (control) included intact rabbits (n=17) maintained on standard vivarium diet, and group 2 (n=18) consisted of cholesterol-treated animals. Fragments of the brain with pia mater at the level of vertical cortex were fixed routinely for light and electron microscopy [8,10]. For light microscopy paraffin sections were stained with hematoxylin and eosin; semithin sections were stained with methylene blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a TESLA BS-500 electron microscope.

RESULTS

Light microscopy revealed no changes in the pial arteries of control rabbits. Endothelial cells (EC) with moderately basophilic round-oval nuclei and thin internal elastic membrane (IEM) were seen in the tunica intima; the tunica media consisted of two or three layers of smooth muscle cells arranged in a circular fashion and a poorly developed tunica adventitia gradually transforming to the connective tissue of the pia mater. In HC, the walls of pial arteries were thickened, loose, edematous, with light spaces under IEM and signs of perivascular edema. The EC nuclei were pyknotic, thickened, sometimes swollen, light, vertically oriented towards the vessel lumen; desquamation of EC was noted in some areas. Marked vacuolization of the brain indicated its edema.

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