QUANTITATIVE EVALUATION OF THE PHAGOCYTIC ACTIVITY OF ENDOTHELIAL CELLS IN CULTURE

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UDC 612.3-08

KEY WORDS: endothelial cells; phagocytosis; fluorescent microspheres.

Endothelial cells (EC) of blood vessels play the role of a selective barrier between the blood and tissue fluid. Depending on the functional activity and integrity of the endothelium, changes take place in the spectrum of proteins capable of passing through the vessel wall. According to some investigators, the initial factor in the development of atherosclerosis is not injury to the epithelial lining or its desquamation, but a disturbance of its function [2, 5, 6]. Hence the need to elucidate the character of endocytosis in EC as a possible mechanism of protein transport across the endothelial barrier.

One method of endocytosis, namely phagocytosis, has received little study in EC. There have been only a few investigations which have demonstrated the ability of EC to phagocytose solid particles [3, 7, 9]. These investigations have shown that the phagocytic activity of EC in the zone of an atherosclerotic lesion is higher than in neighboring areas [7]. However, visual assessment of phagocytic activity is subjective, whereas quantitative analysis would enable not only EC in different parts of the vascular bed to be compared, but the degree of activity of phagocytosis also to be established.

The aim of this investigation was to develop a method of quantitative evaluation of the phagocytic activity of EC from the human umbilical vein in primary culture.

METHODS

EC were isolated from the human umbilical vein with a 0.1% solution of collagenase (Sigma, USA) as described previously [1]. Cells were seeded in a density of $5 \cdot 10^4$ to $8 \cdot 10^4/cm^2$ on a culture plate (Lostar) with wells 16 mm in diameter, or on coverslips. The cells were cultured in medium 199 containing 10% embryonic calf serum, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco, USA) at 37°C in an atmosphere with 5% CO_2. The experiments were carried out on the 3rd to 4th day, or when the effect of the phase of growth on phagocytic activity of EC was investigated, on the 1st to 9th day of culture. Fluorescent carboxylated microspheres (FCM), 0.26 and 0.6 μ in diameter (Polyscience), were used as particles for phagocytosis. The culture of EC was washed three times with 1 ml of Dulbecco's phosphate buffer (DPB, from Gibco). Into each well were introduced 0.5 ml of fresh medium and 0.1 ml of FCM, diluted in DPB. The cells were incubated with FCM for 1 h at 37°C. As the control, some cells were kept with FCM at 4°C or 30 mM sodium azide or 10 μg/ml of cytochalasin B was added to the medium, and the sample was incubated at 37°C. At the end of incubation the EC were washed 7-8 times with cold DPB and treated for 3 min with a 0.25% solution of trypsin with 0.02% EDTA, warmed to 37°C. The cell suspension was diluated with 20 volumes of DPB and centrifuged at 400 g for 10 min. The residue was resuspended in 1 ml of DPB and the intensity of fluorescence measured on an SPF-500™ spectrofluorometer (Aminco, USA). The protein concentration in each sample was determined by Lowry's method [4].

Phagocytic activity of EC was determined as the difference between maximal intensity of fluorescence of cells incubated with FCM at 37 and 4°C, with calculation of relative fluorescence for 10 and 100 μg protein. To monitor the phagocytic activity of EC visually cells grown on coverslips were used. After incubation of EC with FCM for 1 h at 37 or 4°C the cells were fixed with methanol for 10 min, then treated with chloroform for 5 min to remove

Laboratory of Cell and Tissue Culture, Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 101, No. 6, pp. 728-730, June, 1986. Original article submitted January 22, 1985.

 0007-4888/86/0006-0818$12.50

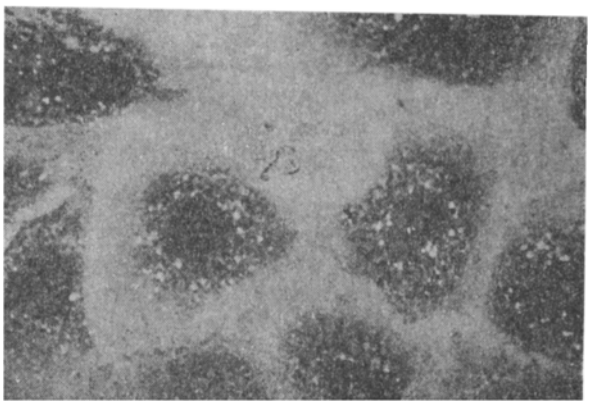

Fig. 1. Distribution of phagocytosed FCM particles in EC from human umbilical vein. EC were incubated with FCM 0.26 μ in diameter for 1 h at 37°C. UV light and phase contrast. Hematoxylin. 500×.

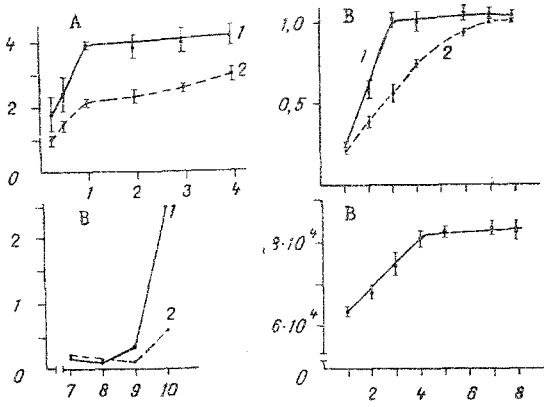

Fig. 2 Fig. 3

Fig. 2. Phagocytic activity of EC depending on dose of FCM (B) and duration of their incubation with cells (A). Abscissa: A) duration of incubation (in h); B) concentration of FCM (number of particles in 1 ml); ordinate, relative intensity of fluorescence (in relative units per 10 and 100 μg protein respectively). 1) EC incubated with FCM at 37°C; 2) control (4°C).

Fig. 3. Dynamics of phagocytic activity (A) and increase in number of EC (B) at various times of culture. Abscissa, duration of culture (in days); ordinate: A) relative intensity of fluorescence (in relation units/10 μg protein); B) number of cells per cm^2. Remainder of legend as to Fig. 2.

nonspecifically bound FCM particles [8]. The preparations were mounted in glycerine and examined under the "Opton" microscope.

RESULTS

Both types of FCM had standard values of fluorescence: with excitation of 450 nm maximal fluorescence was determined at 480-482 nm. When different dilutions of FCM were measured

it was found that the intensity of fluorescence was directly proportional to the FCM concentration in the solution. The fluorescence spectrum remained unchanged on analysis both of a suspension of FCM in DPB and of a suspension of cells containing FCM, or a lysate of these cells. All subsequent measurements were accordingly carried out on cell suspensions.

Values of the intensity of fluorescence of EC during incubation for 1 h with FCM 0.6 μ in diameter, in a dose of $5 \cdot 10^9$ particles/ml, were virtually identical in the experiment and control. Visual analysis revealed only single FCM in individual EC, evidence of an exceptionally low level of phagocytosis. Cells incubated with FCM 0.26 μ in diameter in the same dose for 1 h at 37°C had an intensity of fluorescence 2 or 3 times greater than at 4°C. Visual analysis of these cells showed that in each cell there were 20 FCM or more (Fig. 1). All subsequent experiments were carried out with FCM 0.26 μ in diameter.

The phagocytic activity of EC varied depending on the time of their incubation with FCM and on the dose of FCM. For instance, in experiments in which cells were kept with FCM in a dose of $5 \cdot 10^{10}$ particles/ml at 37°C the intensity of fluorescence increased actively during the first 60 min of incubation, whereas at later times of the experiment it did not change significantly (Fig. 2A). The intensity of fluorescence in the control, in cells kept with the same dose of FCM at 4°C, was appreciably weaker, although it increased in the course of the experiment, indicating an increase in nonspecific sorption. A similar time course of phagocytosis in EC also was observed when they were incubated with smaller doses of FCM. High doses of FCM stimulated phagocytosis in EC, although in that case nonspecific sorption also was increased (Fig. 2B). The same rule was o-served in four experiments, although the dose stimulating phagocytosis varied for each culture.

The phagocytic activity of EC depended on the phase of growth of the culture. In the experiment in which cells were incubated with FCM in a dose of $5 \cdot 10^9$ particles/ml for 1 h, it was found that the phagocytic activity of EC on the first day of culture was very weak, but later it rose, to reach a maximum on the 3rd day of the experiment (Fig. 3A). During this period cells in culture actively proliferated (Fig. 3B). During the later stages of culture the intensity of phagocytosis diminished in EC, and the decrease was especially marked on monolayer formation.

The results thus confirm those of previous investigations [3, 7, 9] in which the ability of EC to phagocytose solid particles such as colloidal carbon and latex was demonstrated. In our view, the use of FCM as the object of phagocytosis has many advantages: They are chemically inert, standard in size, and their intensive fluorescence facilitates visual monitoring ofphagocytosis. Quantitative evaluation of phagocytic activity of EC had helped to bring to light several new facts. For instance, EC actively phagocytosed only small particles, not more than 0.26 μ in diameter. Phagocytosis proceeded rapidly in these cells during the first 60 min of their incubation with FCM, but later during the experiment the number of phagocytosed particles showed very little change and nonspecific sorption of FCM by the cells increased. High doses of FCM stimulated phagocytosis in EC. Phagocytic activity of EC was particularly high in the phase of growth of the culture in proliferating cells, but minimal during the formation of a cell monolayer. Activation of phagocytosis in proliferating EC may be interesting for an understanding of the pathogenesis of development of atherosclerosis.

The quantitative method of evaluation of phagocytosis can be used both to compare the phagocytic activity of different cells in culture and for testing pharmacologic preparations.

The authors are grateful to T. L. Bushueva, on the Staff of the Institute, for help with the use of the spectrofluorometer.

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