BIOPHYSICS AND BIOCHEMISTRY

Development of a Model System for Evaluation of Human Endothelial Cell Proliferation *in Vitro*: Possible Clinical Applications

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 129, No. 4, pp. 404-407, April, 2000
Original article submitted July 28, 1999

A system for evaluation of the ability of human blood serum to affect endothelial cell proliferation was developed and tested. The system based on incorporation of ^{3}H-thymidine into DNA was used to analyze the effects of hormone replacement therapy on endothelial repair and angiogenesis. Blood serum from 12 menopausal women less effectively activated endothelial proliferation compared to control donor serum. After 6-month hormone replacement therapy with Divina (a combination of estradiol and medroxyprogesterone), this index increased in seven female subjects (58.3%), but remained below the control level. The model proposed by us can be used in clinical practice and drug testing for evaluation of the influence of blood serum on vascular endothelium.

Key Words: *endothelial cells; proliferation; test system; angiogenesis*

Endothelial cells (EC) lining blood vessels form a functionally active blood-tissue barrier and are involved in physiological and pathological processes, such as inflammation, angiogenesis, atherosclerosis, and oncogenesis [3,5,11,16]. The repair capacity of the endothelium, *i.e.* recovery of its damaged fragments, is important for its normal functioning [10]. The development of collateral coronary vessels was shown in patients with angina pectoris and acute myocardial infarction [4], as well as in experimental animals [2]; this limited the infarction zone and improved the outcome of the disease [6]. An analysis of the proliferative activity of EC provides estimates of the ability of intimal recovery and budding of new vessels, and compensatory formation of collaterals.

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The repair capacity of the endothelium can be estimated *in vitro* by the proliferation rate of a standard EC culture in the presence of human blood sera or various biologically active substances (drugs, hormones, growth factors, *etc.*) [3,9,11,13]. This approach allows to evaluate the state of endothelium and study the pathogenesis of various vascular diseases. Of special importance is the control over angiogenesis and vascular remodeling in response to endothelial damage in atherosclerosis, complications of diabetes mellitus, myocardial infarction, *etc.*

The use of pharmacological agents activating EC proliferation and thereby accelerating the formation of collaterals, is a promising method of therapy for ischemic heart disease. In light of this, it is important to provide an adequate and reliable model for determination of proliferative activity of the endothelium exposed to various modulating influences.

The goal of this work was to design and standardize a test system based on cultured EC and to evaluate its suitability for biomedical studies.

0007-4888/00/0004-342$25.00

Previous *in vitro* experiments showed that angiogenic activity of the endothelium is regulated by sex hormones, primarily estrogens [11,14,15]. These hormones are important for regulation of lipid metabolism, including the metabolism of cholesterol [15]. Here, we evaluated the ability of blood serum taken from postmenopausal women before and after 6-month hormone replacement therapy (HRT) to affect EC proliferation.

MATERIALS AND METHODS

Primary cultures of EC were isolated from five human umbilical cords by perfusion with 0.2% collagenase (Serva) as described previously [8,10]. Cultured cells were maintained on a standard complete growth medium [11,13] containing DMEM, 50 μg/ml ECGS, and 20% embryonic calf serum (both from Sigma). The cells were plated in 35-mm Petri dishes (Costar) precoated with 0.2% gelatin and passaged using 0.25% trypsin-0.02% EDTA (Gibco). The purity of the EC culture was verified by a typical monolayer pattern (cobblestone), the presence of factor VIII, and absence of smooth muscle cell markers (α-actin and myosin) by immunochemical test [13,16] with specific monoclonal antibodies (Sigma).

Proliferative activity of EC (second and third passages) was tested in cells seeded in 35-mm 6-well plates (Costar) at a density of 2×10^4 cells/cm^2. The cells were cultured for 72 h to 75% confluency. The culture was then synchronized by transferring the cells into G_0 phase in inhibition medium containing phenol-free DMEM, 2% embryonic calf serum, and 0.5% BSA. Phenol was excluded from the medium to prevented its effect on EC, which is similar to the effect of estrogens [11]. After 48 h, the cells were washed with phosphate-buffered saline, and test medium was added: phenol-free DMEM containing 20% human serum without growth additives. Complete growth medium was used as the positive control, negative control tests were performed with inhibition medium, and reference samples were placed in growth factor-free medium containing 20% pooled blood serum from 20 healthy female donors. EC proliferation was determined quantitatively by incorporation of ^{3}H-thymidine into DNA. Cell protein was determined by Bradford's micromethod (Biorad). Cell fractions precipitated with 10% trichloroacetic acid (TCA) were dissolved in 0.3 ml 0.1 N NaOH. The label incorporation was measured in 100 μl of this solution, and protein was determined in 150 μl. Proliferation was expressed in cpm/μg cell protein.

The feasibility of this system for clinical tests was examined by using serum samples from 12 postmenopausal women with hypercholesterolemia (>200 mg/dl). The tests were performed before and after 6-month replacement therapy with Divina, a two-phase hormone product of Orion Pharmaceutica (the administration scheme of estradiol and combination of estradiol with medroxyprogesterone).

All serum samples and pooled standard were inactivated by heating at 56°C for 30 min, filter-sterilized (0.45 μ), and stored at -20°C before the test [13].

The state of the lipoprotein system was determined by measuring total serum cholesterol and triglycerides on a Centrifichem-600 automatic analyzer by enzymatic methods with Randox kits. HDL cholesterol was determined by the same method after sedimentation of apolipoprotein B-containing lipoproteins with phosphotungstic acid [1].

The data were processed by Wilcoxon matched-pairs test and Student's t test at $p<0.05$.

RESULTS

A number of methods for estimation of EC proliferation were described: cell counting, determination of DNA by fluorescent dye binding, and incorporation of ^{3}H-thymidine into DNA [3,9,11,15,16]. The method of ^{3}H-thymidine incorporation chosen by us because of its accuracy and adequacy is most frequently used in cell studies.

Optimal conditions of the test were found by varying the time of cell incubation in test medium (48, 72, and 96 h), comparing the label incorporation (1 μCi/ml) over 18 h in test medium with pulse-chase labeling (2.5 μCi/ml) for 2 h in a serum-free medium, and estimating the percentage of TCA-insoluble frac-

TABLE 1. Proliferation Responses (cpm/μg Cell Protein) of Endothelial Cells at Various Terms of Incubation with Test Medium (*M*±*m*)

Medium	Incubation time, h		
	48	72	96
Positive control (complete growth medium)	15,134±298	4123±78	545±34
Reference samples (20% pooled serum)	407±75	320±16	212±14
Negative control	195±17	187±20	190±12

TABLE 2. Effects of Hormone Replacement Therapy on EC Proliferation Response to Human Blood Serum (Reference Sample, 407 cpm/μg Protein)

Patient No.	Before therapy	After therapy
1	115	122
2	125	120
3	127	148
4	126	199
5	170	146
6	180	175
7	173	202
8	206	234
9	230	280
10	282	315
11	324	350
12	340	375

tion by means of EC fixation directly to the plate or incubation of trypsin-treated spun-washed EC with TCA.

The peak of EC proliferation was observed 48 h after adding complete growth medium or medium containing human blood serum (Table 1). Cell proliferation in complete growth medium far surpassed a relatively weak and prolonged response to 20% human serum. The concentration of growth factor used for *in vitro* EC culturing is much higher than its concentration in human blood serum *in vivo*, which most probably explained the more than 37-fold difference in the proliferation rate. These data are consistent with previous reports on relatively low proliferative activity of human EC *in vivo* [5,12,15].

Pulse-chase labeling in a serum-free medium for 2 h was more informative than long-term (18 h) incubation in test medium: 15,134±298 *vs.* 8523±184 cpm/μg in complete growth medium ($p<0.05$). Similar data were obtained in experiments with pooled reference sample (407±75 and 310±56 cpm/μg, respectively); however, this difference was insignificant, probably due to adsorption of ^{3}H-thymidine by the serum or quenching of cell proliferation during long-term incubation.

Homogeneous cell suspension obtained by trypsinization was more suitable for manipulations. This treatment prevented cell loss and decreased variations of the indices of proliferation response to 12.6% *vs.* 24.5% (sometimes 30%) for attached cells.

Hence, optimal conditions are the follows: incubation of cells in test medium for 48 h, pulse-chase labeling, and trypsinization of EC monolayer before fixation with TCA. This scheme was used in further tests. Experiments with EC cultures isolated from various donors showed that major control indices varied between tests by no more than 5-7%, that is, the system yielded highly reproducible results.

An analysis of the effects of blood sera from 12 postmenopausal women on EC cultured under standard conditions (Table 2) showed that before HRT the proliferation index was below the reference level in all patients (184±66 and 407±75 cpm/μg, respectively, $p<0.01$). Moreover, the sera from 10 (83.3%) patients inhibited EC growth. After HRT, the EC proliferation index increased significantly ($p<0.05$) in 7 (58.3%) patients (Table 2), but remained below the reference level. It should be noted that despite group homogeneity by sex, age, hormonal state, and the level of total cholesterol (more than 200 mg/dl), proliferation activity of test sera considerably varied (from 340±14 to 82±7 cpm/μg). Label incorporation before and after HRT differed by 21% on average (the maximum change was 54.7%). Taken together, these data suggest that adequate evaluation of the ability of a patient's serum to modify EC proliferation requires repeated measurements of this parameter after long time intervals (several weeks or months).

Hormone replacement therapy slightly decreased total serum cholesterol (from 269.0±14.1 to 239.0± 19.7 mg/dl, by on average 10%, $p<0.05$). These data suggest that the decrease in cholesterol level after hormone replacement therapy correlated with the ability of blood serum to stimulate EC proliferation *in vitro*.

Thus, we developed, standardized, and tested a cell system for evaluation of the effect of blood serum on EC proliferation *in vitro*. The system is simple, reproducible, and sensitive; it may be used for evaluation of direct effects of biologically active substances on EC and examination of patients after therapy, surgical interventions (recovery of damaged blood vessels), or patients with solid tumors.

We are grateful to Prof. N. V. Perova, Head of Department of Biochemistry, State Research Center of Preventive Medicine, for fruitful discussion and valuable comments.

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