Comparative Study of the Effects of Trophoblastin and Polydeoxyribonucleotide Preparations on Cultured Skin Fibroblast

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We studied the effects of trophoblastin and polydeoxyribonucleotide on the morphology and functional characteristics of fibroblasts in the culture of skin cells. Positive effects of trophoblastin and polydeoxyribonucleotide on fibroblast growth and survival during culturing were demonstrated. Trophoblastin exhibited similar effects on cells of early and late passages. Polydeoxyribonucleotide exhibited a more pronounced effect on cells of late passages, whose growth activity decreased with age. Being a multicomponent preparation, trophoblastin stimulated processes leading to positive dynamic of cell development and vital activity. Polydeoxyribonucleotide had a favorable impact on functioning of basal cell structures at the level of cell nucleus and maintained high level of cells with complete DNA set.

Key Words: *trophoblastin; polydeoxyribonucleotide; fibroblasts; aging*

Great attention of specialists is focused on studies of processes underlying skin aging and creation of drugs stimulating regression of age-related changes. Specific features of natural aging [2] suggest that this process can be inhibited; in addition, there is a possibility of creating drugs capable of prolonging optimal functioning of body cells or even attaining the effect of cell rejuvenation (return to their younger status). This requires long and serious studies. The first phase of these studies is evaluation of the effects of activator/regenerative agents on organs and various cell types.

The skin carries information about the health status and largely determines general appearance of the individual. Of all types of skin cells, fibroblasts are responsible for general status of the skin, normal function, supply with necessary biological components, general appearance, *etc.* [10].

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Normally, cells in culture pass a certain number of divisions (similarly as in the body). Cell life *in vivo* and *in vitro* differs significantly, but the processes running in the cells in both cases are similar [7]. When we know how cells behave in culture, we can simulate the processes taking place in the skin, both young and mature.

The study of the mechanisms underlying the effects of drugs on fibroblast aging in skin cell culture and clearing out the essence of the aging process will help us to develop new approaches inhibiting age-associated changes in skin fibroblasts.

Creation of drugs improving skin cell status to a level typical of young growing organism seems to be ideal [5]. However, this should not be associated with deterioration of other characteristics. In this case we can hope that the positive effects of these preparations determined by improvement of the general status of tissue and cell structures will persist for a long time even after discontinuation of treatment.

Here we studied the effects of trophoblastin (TB) and polydeoxyribonucleotide (PDRN) on the status of fibroblast population in skin cell culture: their viability, morphology, and functional activity. Trophoblastin is an extract of human trophoblast fetal cells and umbilical cord mesenchymal cells containing a complex of bioactive proteins and peptides. Polydeoxyribonucleotide is an extract of the trout sex cells (milt) with high content of polynucleotides (DNA); from chemical point of view, it is a linear polymer consisting of deoxyribonucleotide residues connected via phosphodiester bonds.

There are published data [9,11] on positive effect of PDRN on fibroblast growth and function. Pilot experiments demonstrate similar effects of TB. It seems interesting to detect their common positive effect and its mechanisms, to study the effects of the test preparations on cell status at different stages of their vital and functional activity, and to determine possible algorithm of optimization of this effect and its maximum prolongation.

MATERIALS AND METHOD

The study was carried out on a culture of skin fibroblasts isolated from fetal material (11-12-week gestation) obtained from Moscow hospitals. Culturing was carried out by the standard methods. Cells were isolated by trypsinization of skin specimens and cultured in DMEM with 10% bovine serum.

Confluent cultures were reinoculated after 3-4 days (1:2, 1:3) into new flasks or used in experiments. To this end, the cells were treated with a mixture of 0.25% trypsin and 0.02% Versene (1:1 dilution). The seeding density was 30,000-40,000 cell/cm^2. Morphological studies and cell counting were carried out under a Leica inverted microscope using a measuring grid.

TB was isolated from human placental and umbilical cord tissues. To this end, the trophoblast was mechanically homogenized with two freezing-defrosting cycles, extracted in an equivalent volume of water, and centrifugated. The extract was standardized by protein concentration.

TB was fractionated by HPLC on a Toyopearl HW-60 carrier. The fractions with equal molecular weight were pooled (fraction 1: high-molecular-weight protein complexes of ≥500-1000 kDa; fraction 2: proteins and protein complexes of 100-500 kDa: fraction 3: proteins and protein complexes of 50-100 kDa; fraction 4: proteins and protein complexes of 10-50 kDa; fraction 5: low-molecular-weight proteins and large peptides of 1-15 kDa; fraction 6: peptides, amino acids, sugars <5 kDa). Resultant fractions were analyzed by electrophoresis after Laemmli in denaturing 12.5% polyacrylamide gel under non-reducing conditions.

The effects of different TB fractions on fibroblasts in skin cell culture were evaluated by survival of passage 5 cells after addition of each fraction into culture medium. Protein content of 0.15 mg/ml was taken for a concentration unit for all fractions; the fractions were added to cell cultures in 1:4 dilution.

PDRN preparation (Placentex, Mastelli Laboratory) was a gracious gift from VIP Clinic Corporation.

TB and PDRN were diluted 1:1 with serum-free DMEM and then diluted 10-, 100-, 1000-, and 10,000-fold. The volume of sample containing the medium with cells and the studied preparation was 500 µl. Each dilution was tested in 4 parallels. The control sample contained an equivalent volume of culture medium.

In order to detect differences in the morphology of "old" cells treated with TB and PDRN (1:100 dilution), cell distribution according to DNA status was evaluated by standard flow cytometry [6]. For the analysis, the cells were stained with Hoechst 33258 in 0.1 M Tris-HCl (pH 7.2, 0.1 NaCl).

The study was carried out in two model systems: "early" (passage 5) and "late" (passage 17) cells. Human embryonic skin cells of passages 5 and 17 were cultured at a density of 55,000-65,000/cm^2 in 24-well plates (Nunc) in serum-free DMEM. Cells were counted 48 h after addition of the test preparations. The effects of individual TB fractions were studied on skin fibroblasts of passage 5.

RESULTS

Addition of TB and PDRN to early passage cultures resulted in similar growth stimulation (Table 1). Presumably, a common effect is observed during this active phase of growth and proliferation: both preparations are involved in cell metabolism, improve and stimulate it. The growth potential is highly expressed in a young organism, similarly as in early passage cells; the appropriate factors and active substances essential for cell growth and functioning are actively produced and accumulate forming a sort of "inner reserve". For this reason, the growth potential of cells is well expressed in the control (without studied substances) and experimental (with preparations) samples. We can assume that during these passages the effect of TB stimulation is less concentration-dependent in comparison with PDRN effect. It seems to be due to complex composition

of TB and hence, mutually "overlapping" and presumably potentiating effects of its components.

The effects of the test preparations on "old" cells of late passages are somewhat different (Table 2). On the whole, PDRN effect is similar to its effect on the early passage cells, while the effect of TB under these conditions is more active, which manifests by a greater difference between the values in experimental and control groups. A reduction of positive effect was observed in the late passage cells in the presence of high PDRN concentration (1:1), which can presumably be explained by the effect of medium dilution, most pronounced for late passages, when the cell potential is reduced and their activity much more depends on external factors and quality of medium than during early passages.

Cell DNA status is one of the main indicators of its viability [8]. Preliminary analysis revealed a certain relationship between cell DNA status and treatment with different preparations. The percentage of cells with normal DNA set increased in some experiments under the effect of PDRN. It was presumably due to specialized composition of the preparation, its main component being the "construction" material, essential for the synthesis and replication of DNA chains. Thorough selection of certain PDRN fractions by the producer company seemed to be also important, because it resulted in optimal combination of its characteristics. On the one hand, the preparation effectively penetrated into intercellular space, penetrates through membranes, barriers of different kinds, and enters the needed site in the intracellular space; on the other hand, the components penetrated into the cell in the form requiring no serious expenditures for their incorporation in the synthesized or restored chains.

The use of TB increased number of cells in the active phase. The DNA status in this case negligibly differed from the control. The effect of TB on cells was similar to the effect of a multicomponent stimulating serum, added to the culture, and simultaneous increase of the nutrient concentrations. Hence, its effect is polyfunctional, consisting of a sum of effects caused by a complex of active substances in its composition (growth factors, glycoproteins, interleukins, *etc.*), stimulating the processes leading to positive dynamic in the develop-

TABLE 1. Effects of TB and PDRN on the Growth of Skin Fibroblasts during Passage 5

Dilution	Cell count, $\times 10^4/cm^2$			
	TB		PDRN	
	per visual field	% of control	per visual field	% of control
Control	5.660±1.679	100.0	5.970±1.193	100.0
1	8.78±4.095	155.0	9.62±3.303	161.1
1:10	7.42±3.421	130.8	7.15±1.843	119.7
1:100	6.09±1.434	107.3	7.83±3.041	131.2
1:1000	6.27±1.256	110.7	6.920±1.669	115.9
1:10 000	6.840±1.945	120.8	5.930±1.182	99.2

TABLE 2. Effects of TB and PDRN on the Growth of Skin Fibroblasts during Passage 17

Dilution	Cell count, $\times 10^4/cm^2$			
	TB		PDRN	
	per visual field	% of control	per visual field	% of control
Control	2.540±0.780	100.0	2.900±0.727	100.0
1	5.660±2.109	222.3	3.290±1.338	113.4
1:10	4.790±1.892	188.1	4.270±0.943	147.2
1:100	4.490±1.339	176.1	4.040±0.826	139.0
1:1000	4.86±1.712	191.0	4.230±1.012	145.9
1:10 000	3.460±1.309	135.8	3.540±0.816	121.9

ment and vital activity of cells. The possibility of regulating cellular parameters by means of bioactive substances of protein/peptide nature was described [1,3].

In order to study the constituents of multicomponent effect of TB, the preparation was fractionated. The problems concerning the active fractions (whether all fractions were active), type of their effect, and presence of ballast fractions impeding the functioning of active components were studied. The elution profile of TB protein-peptide complexes is presented (Fig. 1). Fractions obtained after fusion were analyzed by electrophoresis. Protein and peptide content of fractions 2, 3, and 4 was maximum, while the rest fractions contained negligible concentrations of protein components.

Survival of passage 5 fibroblasts under the effects of fractions isolated after TB separation, are presented in Fig. 2.

Virtually no properly functioning cells were seen in a medium without TB fractions as soon as on day 5, which indicates significant positive effects of individual fractions and the preparation on cell quality. Fractions 1-4 exhibited the greatest effects on cell survival and growth; it seems that these fractions contain the maximum levels of growth factors. On the other hand, it is known that the majority of growth factors are polypeptides with a molecular weight of 5-15 kDa (which corresponds to fraction 5), but just a minor part of them can occur in a free form [4]. The major part of factors (up to 98%) are bound and aggregated, and in this form their molecular weight reaches several tens kDa, which corresponds to the active fractions. Obviously, fraction 6 contains virtually no growth factors, and the improvement of cell parameters in the presence of this fraction is most likely due to additional nutrients received by cells.

The effect of TB fractions on cell survival and growth suggests that fraction 6 contains mainly nutrients and metabolites essential for the maintenance of normal cell functioning, fractions 3 and 4 contain growth factors, fractions 2 and 5 contain growth factors and metabolites, fraction 5 presumably contains pure growth factors and metabolites, and fraction 2 most likely contains them in the form of aggregates and in partially adsorbed form. Fraction 1 contains small proteins with high molecular weight, on which growth factors and other substances essential for active cell functioning are adsorbed. The profile of time dependence (maximum values were recorded during later periods in comparison with other fractions) indicates that the release of active components into the medium is gradual, protracted in time.

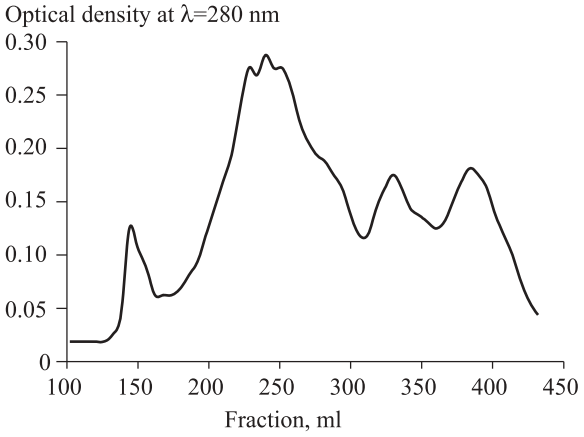

Fig. 1. Elution profiles of protein-peptide complexes obtained during TB separation.

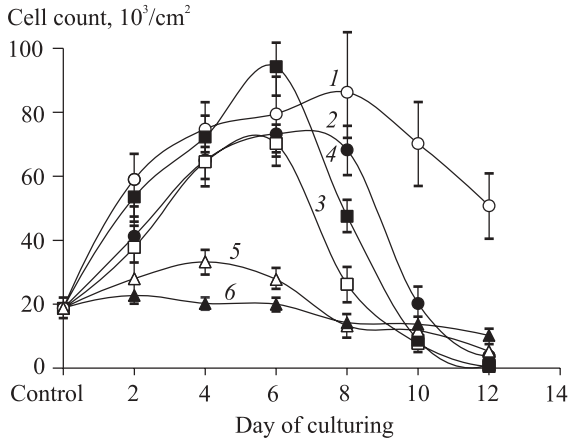

Fig. 2. Survival of skin fibroblasts in passage 5 under the effects of fractions isolated after TB separation.

The results of mathematical processing of cell survival curves under the effect of TB fractions indirectly confirm these hypotheses. Starting from the low-molecular-weight fractions, the curve equations are becoming more complex, describing the results of experiments with the maximum approximation. The curves presenting the effects of fractions 5 and 6 are described by the 3rd degree polynome, while fraction 1 curve is described by the 5th degree polynome. It is most likely explained by the fact that with increasing the molecular weights of the components, the effects of fractions become more and more complex and multifactorial.

The increase of cell growth in response to TB complex is mainly due to acceleration of the work of all cell systems. The increment in cell growth in this case is attained due to improvement of the qualitative characteristics of intracellular matrix and intracellular contents, specifically of its consumed part, as the cells are more actively supplied with construction and signal substances. Cell growth in-

crease does not at all modifies their inner potential and hence, does not lead to its exhaustion and requires no forced measures for its restoration in future.

Hence, the positive effects of the test preparations on skin cells can be mediated by two different mechanisms. PDRN modulates cell functioning at the level of cell nucleus maintaining high level of cells with complete DNA set. Multicomponent preparation TB stimulates processes of different nature, and its effects cover a wide range, from trivial supply with growth-limiting substances to improvement of intracellular regulation, which leads to positive dynamic of cell development, vital activity, DNA and protein synthesis. Positive effect of TB is balanced and variegated. Different TB fractions contain growth factors, nutrients essential for the cell, cell regulators, and other metabolites. High-molecular-weight fraction of the preparation also plays a positive role. Due to this constituent the effects of TB can be prolonged and the preparation components partially located, which largely substitutes for the functioning of the so-called "transporting systems" used for improving the efficiency of bioactive preparations.

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