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Adhesion, Growth, and Proliferation of Endothelial Cells on Biopolymer Extracellular Film Matrices

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We analyzed the influence of initial properties of biopolymer film extracellular matrices made of chitosan on adhesion of swine embryonic kidney epithelium-like cells, their morphology, growth, and proliferation. It was found that adhesion index of cells on the studied matrices was similar, but cell morphology had significant qualitative differences. Round non-flattened cells were described in endothelial cell cultures on chitosan-based matrices. Local variations in charge density on matrices induced by the presence of chitin nanofibers led to the appearance of flattened spindle-shaped and non-flattened sphericalal cells. Long-term culturing on biopolymer extracellular film matrices was associated by the formation of spatial conglomerates of sphericalal cells.

Key Words: *adhesion; proliferation; biopolymer extracellular film matrix; chitosan; chitin nanofibres*

Extracellular matrix (ECM) is the basic component of tissue and organic architectonics. Micro- and nanopathology of ECM, variability of the chemical structure of biomolecules, and their proportions and mutual arrangement determine diversity of ECM structures essential for normal functioning of different cell types [21]. The main structural components of cell interacting with ECM are transmembrane receptor proteins of the integrin family. Integrins participate in cell–cell contacts and contacts between the cell and extracellular microenvironment and mediate cytoskeleton–ECM interactions [16]. *In vitro* studies showed that changes in ECM nanotopology modulate focal adhesion of epithelial cells [13]. It has been found that differentiation of neural stem cells into neurons or astrocytes depended on the topology of biopolymer extracellular film matrices (BEFM) [24]. Moreover, environment, namely, conformation and proportions between the main ECM proteins: collagen, elastin, fibronectin, laminin, as well as glycoproteins, glycosaminoglycans and proteoglycans concentration, affect cell behavior during their interaction with the extracellular substrate [15,19].

Chitosan (poly(1→4)2-amino-2-deoxy-β-D-glucan), a polyaminosaccharide product of chitin deacetylation, is characterized by low toxicity, biodegradadability, solutability in weakly acidic water solutions and film-forming potential, which makes it a promising substance for BEFM modeling [22]. A promising approach to modification of natural polysaccharides is creation of composites with nanoparticles of different nature and architectonics. Introduction of nanoparticles into polymer matrix can considerably change the hydrophilic-hydrophobic, stress–strain, and barrier properties of the films and morphology of their surface. The use of natural nanofiller (chitin nanofibers) for chitosan modification preserves positive properties of the matrix polymer (low toxicity, biodegradability). In modern cell transplantology and tissue engineering, the development of new chitosan-based BEFM is the primary goal for the creation of implants of injured organ and tissue [1-3,23]. However, regulation

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of the main processes determining cell behavior during their interactions with BEFM is poorly studied. The study of adhesion, proliferation, differentiation, and apoptosis of cells cultured on BEFM with different spatial geometry and physicochemical characteristics is now the major model to study cell–cell interactions involved in the formation tissue structures both *in vitro* and *in vivo* [14].

Here we studied adhesion, growth, and proliferation of endothelial cell culture on BEFM based on chitosan and its composites with chitin nanofibers.

MATERIALS AND METHODS

For BEFM formation, we used chitosan isolated from crab crusts (Bioprogress). Chitosan deacetylation degree (0.88) was evaluated by conductometric titration. The mean molecular weight determined using a Ubbelohde capillary viscosimeter was 172 kDa. Chitin nanofibers isolated using a modified technology [11] were used as a nanomodifier. For isolation of chitin nanofibers, we used fibrillary crystalline α-chitin. α-Chitin fraction with particle size of 0.1-0.2 mm was subjected to alkaline deacetylation at 90°C for 3 h in 33% NaOH with $NaBH_4$. The product was dialyzed against water and lyophilized. Chitosan deacetylation degree (0.42) was evaluated by conductometric titration. Aqueous 0.1% suspension of chitin nanofibers was mechanically agitated for 5 days and then sonicated for 10 min. After alkaline deacetylation followed by mechanical dispersion and ultrasound processing, stable chitin nanofiber dispersion was obtained. Thickness and length of nanofibers (5-10 and 100-500 nm, respectively) were measured by electron microscopy.

Chitosan films (BEFM-1) were prepared from 3% chitosan in 2% acetic acid by forming from a spinneret on a glass substrate followed by drying at room temperature. Composite chitosan films containing chitin nanofibers (BEFM-2, BEFM-3, BEFM-4, BEFM-5) were made using two different approaches. The first approach implied addition of calculated volume of 0.1% nanofiber dispersion to 4% chitosan in 2% acetic acid followed by agitation for 2 h; the films were obtained by dry formation. The second approach consisted in mixing chitosan powder with 0.1% nanofiber dispersion in water for 2 h and addition of 100% acetic acid to its 2% concentration. The obtained mixture was agitated to dissolve chitosan completely (4% solution) and the films (20-30 μ thick) were prepared by dry formation. Film surface morphology was studied under a Supra 55VP-3249 (Carl Zeiss) scanning electron microscope; film samples were mounted on a conducting carbon substrate and dusted with gold. The films were mechanically tested in a UTS 10 universal test system (UTS Testsysteme).

We used transplantable embryo pig kidney endothelial cell culture (SPEV). SPEV cells were cultured in DMEM/F-12 (Sigma-Aldrich) with 10% FCS (HyClone), 100 U/ml penicillin, 100 U/ml streptomycin, 1.25 mg/ml gentamicin, and 0.5 mg/ml amphotericin B (Sigma-Aldrich). The seeding cell concentration in Petri dishes (d=30 mm) was $1.9\text{-}2.1\times10^5$ cells/ml. The medium was replaced every 3 days. Cell culturing was performed in a CO_2-incubator (Sanyo).

BEFM samples (2.7×2.7 cm; 7.29 cm^2) based on chitosan and its composite with chitin nanofibers were preliminary sterilized in UV light (254 nm) for 30 min, placed to Petri dishes (d=30 mm), incubated in culture media for 5 min, and then the cells were seeded. Control dishes contained no films. Adhesion index for SPEV cells was calculated after 24-h culturing by the formula:

$$I=\frac{A\text{-}B}{A}\times100\%,$$

where A is the initial cell concentration in 1 ml of culture media and B is cell concentration after 24-h culturing [10].

Proliferation index of the culture was calculated as the ratio of cells isolated after 1, 3, and 5 days of culturing to cells seeded. In the control, the culture did not reached confluence at the specified seeding concentration after 5-day culturing. The cells were counted in a Goryaev's chamber.

The morphology of living cells and dynamics of monolayer formation were estimated on days 1, 3, and 6 under an inverted microscope (Olimpus) equipped with Scopetek DCM-130E camera (lens ×20).

The morphology of adherent cells in 1-, 3-, and 6-day culture was studied by scanning electron microscopy. The cell culture was 3 times washed from culture media with physiological saline and fixed in 2.5% glutaraldehyde in phosphate buffer [9]. The cells were dehydrated in ascending ethanol concentrations (50-96%) for 15 min and twice in absolute alcohol (98%). The samples mounted on a grid were tinted with silver in a vacuum evaporator at an angle of 45°. Thickness of silver layer applied to the sample was 20-25 Å. The samples were examined under a REMMA-101A canning electron microscope (Selmi) operated in secondary electron mode at accelerating voltage of 20 kV (×2000-10,000). The images were transmitted to a monitor and processed using SEO ImageLab image analysis software (Sumy Electron Optics).

RESULTS

To study cell adhesion and proliferation, chitosan films and composite (chitosan–nanofiber) films with different concentration of the nanomodifier were prepared

and their stress–strain properties were studied. The elasticity modulus (E), plastic limit σ_n, ultimate stress σ_p, and breaking strain ε_p were determined (Table 1). Chitin nanofibers used as chitosan film modifier change the stress–strain properties of the films; this modification depends on the amount of modifier added and the way of its introduction (Table 1; the first method for BEFM-3, -4, and -5 samples and the second method for BEFM-2). All composite chitosan–chitin nanofiber films showed high strength characteristics and sufficient deformation properties. The composite film made using the second method of chitin nanofiber introduction demonstrate significant increase of elasticity modulus (E) and plastic limit (σ_n) in comparison with the initial chitosan film and films made using the second way of nanofibers introduction. This can be related to active influence of the nanofiller on chitosan matrix structure and probably suggests that chitin nanofibers armor chitosan. Taking into account the complex of stress–strain properties, BEFM-1 and BEFM-2 were chosen as polymer matrices for cell culturing (Table 1). Electron microphotographs of the films show structural heterogeneity of BEFM-2 surface (Fig. 1, *b*) caused by the nanofiller, in contrast to chitosan BEFM-1 film (Fig. 1, *a*) with homogenous low porous surface.

The adhesion and proliferation properties of SPEV cells on BEFM based on chitosan and its chitin nanofibers composites are presented in Figure 2. Adhesion index after 1 day of culturing (Fig. 2, *a*) on BEFM-1 and BEFM-2 was 74.2 and 62.5%, respectively, *i.e.* similar to index obtained after culturing on plastic (76.2%). Analysis of cell proliferation showed different growth on BEFM (Fig. 2, *b*). Cell growth on BEFM-1 was very slow. Proliferation index on day 5 of culturing on BEFM-1 was 2-fold lower than on BEFM-2 and plastic.

Qualitative differences in cell morphology were found after culturing on BEFM-1 and BEFM-2 (Fig. 3). Thus, the cells cultured on plastic were flattened and spindle-shaped after 1 day (Fig. 3, *a*, *d*) and on BEFM-1 they had spherical shape (Fig. 3, *b*, *e*). Culturing on BEFM-2 (Fig. 3, *c*, *f*) led to the appearance of both flattened spindle-shaped cells as in the control and non-flattened spherical cells as on BEFM-1. Increasing culturing time on plastic led to the formation of a monolayer that reached confluence on day 6 of culturing (Fig 4, *a*, *d*), while cells cultured on BEFM-1 (Fig. 4, *b*, *e*) and BEFM-2 (Fig. 4, *c*, *f*) did not form the monolayer.

In these cases, spatial conglomerates of spherical cells were forming on BEFM. On BEFM-1, conglomerates had spherical shape (Fig. 4, *b*) and on BEFM-2 we found a sandwich structure, the lower layer consisting of flattened cells (Fig. 4, *c*). In both cases, the spatial structure consisted of spherical cells.

The obtained results suggest that adhesion, proliferation, cell shape, their flattening, and formation of the monolayer and cell clusters on BEFM-1 and BEFM-2 can be determined by topology of extracellular matrices and initial properties of polymers (chitosan and chitin deacetylation degree, molecular weight, *etc*.). Thus, cell adhesion on BEFM and on plastic was similar, but the rate of cell proliferation on plastic and on BEFM-2 significantly surpassed that on BEFM-1 (Fig. 2). It should be noted that during culturing on

TABLE 1. Mechanical Properties of Chitosan and Chitin Nanofiber Films

Sample	Chitin nanofibers, wt.%	Mechanical properties of the films			
		E, GPa	σ_n, MPa	σ_p, MPa	ε_p, %
BEFM-1	0	4.39±0.13	88±1	143±3	54±4
BEFM-2	1	5.85±0.19	128±3	142±3	21±1
BEFM-3	2	3.97±0.11	79±3	93±4	37±3
BEFM-4	3	3.88±0.21	78±3	91±3	31±2
BEFM-5	5	3.52±0.32	67±11	65±9	17±1

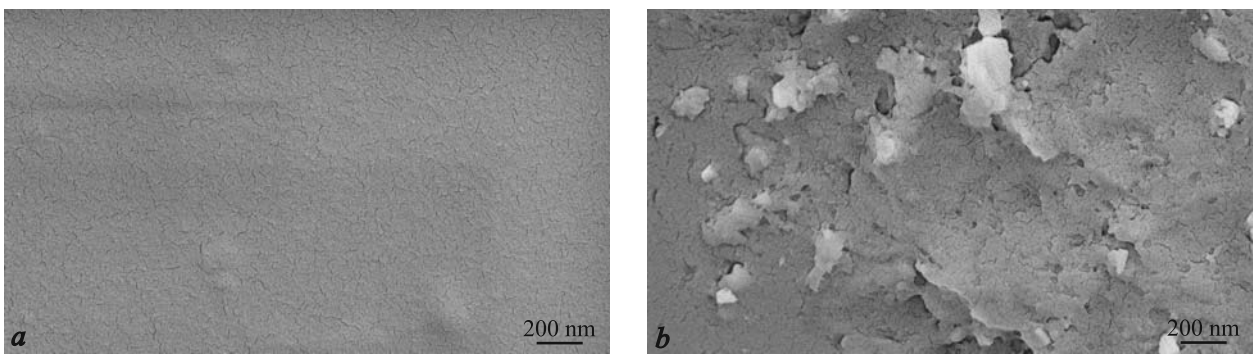

Fig. 1. BEFM-1 (*a*) and BEFM-2 (*b*) films.

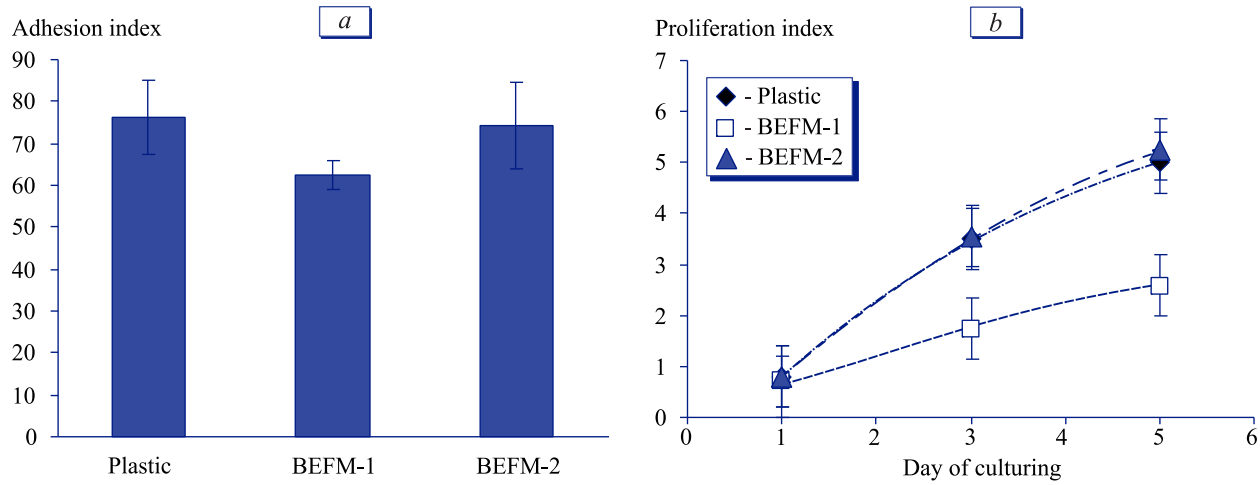

Fig. 2. Index of SPEV cell adhesion (*a*) and proliferation (*b*) during culturing on BEFM.

BEFM-1 no flattened cells were found. The morphology of cells on BEFM-1 was presented by spherical shape (Fig. 3) that is not typical of SPEV cells. The presence of spindle-shaped cells along with spherical cells on BEFM-2 seems to determine higher cell proliferation rates on BEFM-2 in comparison with BEFM-1 (Fig. 2).

The cell behavior on BEFM is determined by "protein-biomaterial" and "cell-biomaterial" interactions. In fact, cell adhesion and growth *in vitro* is preceded by protein adsorption on BEFM [7]. Numerous studies [17, 20] have demonstrated that surface properties of BEFM (hydrophobicity, voltage magnitude and discrecity, roughness) can modulate qualitative and quantitative composition of adsorbed protein molecules and their conformation. During *in vitro* cell culturing, the basic proteins in culture medium adsorb on the substrate. Physicochemical properties of BEFM surface determine absorption of culture medium proteins, their interaction with integrin receptors of the membrane, and structure of focal contacts that transmit signals from matrices to cells and initiate intracellular

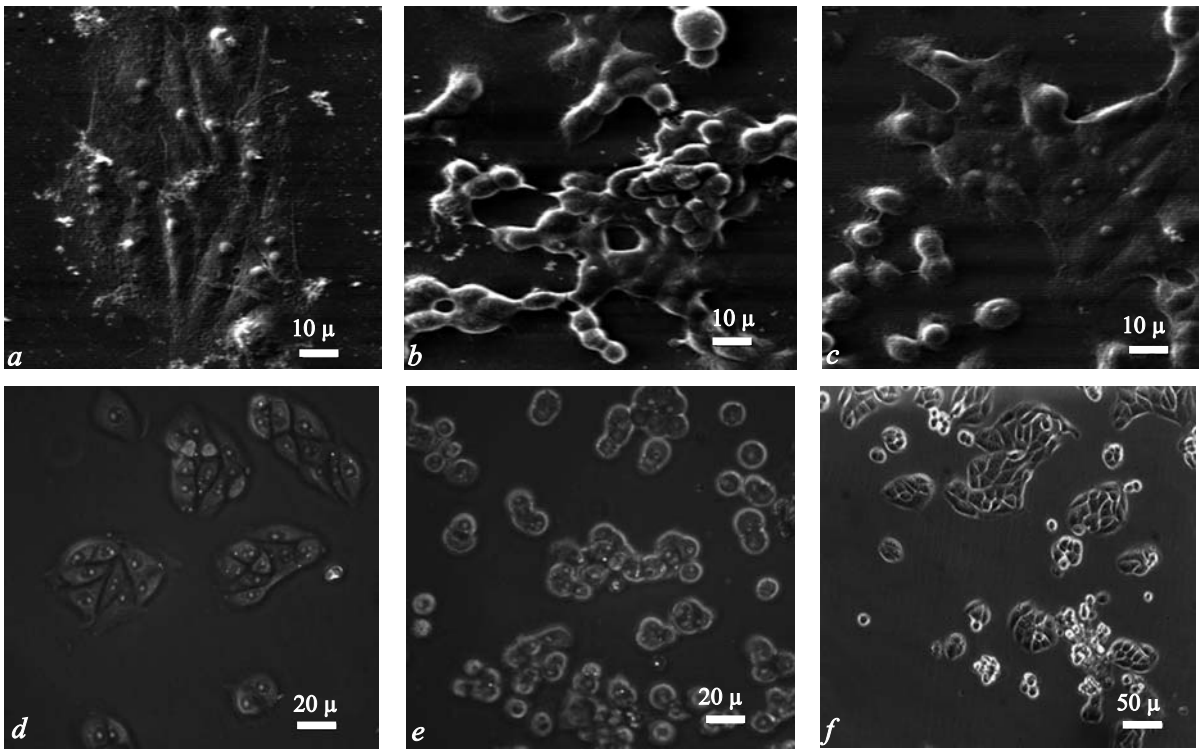

Fig. 3. SPEV cell morphology in 24-h culture. Here and in Fig. 4: scanning electron microscopy (*a-c*) and light microscopy (*d-f*): on plastic (*a*, *d*), on BEFM-1 (*b*, *e*), on BEFM-2 (*c*, *f*).

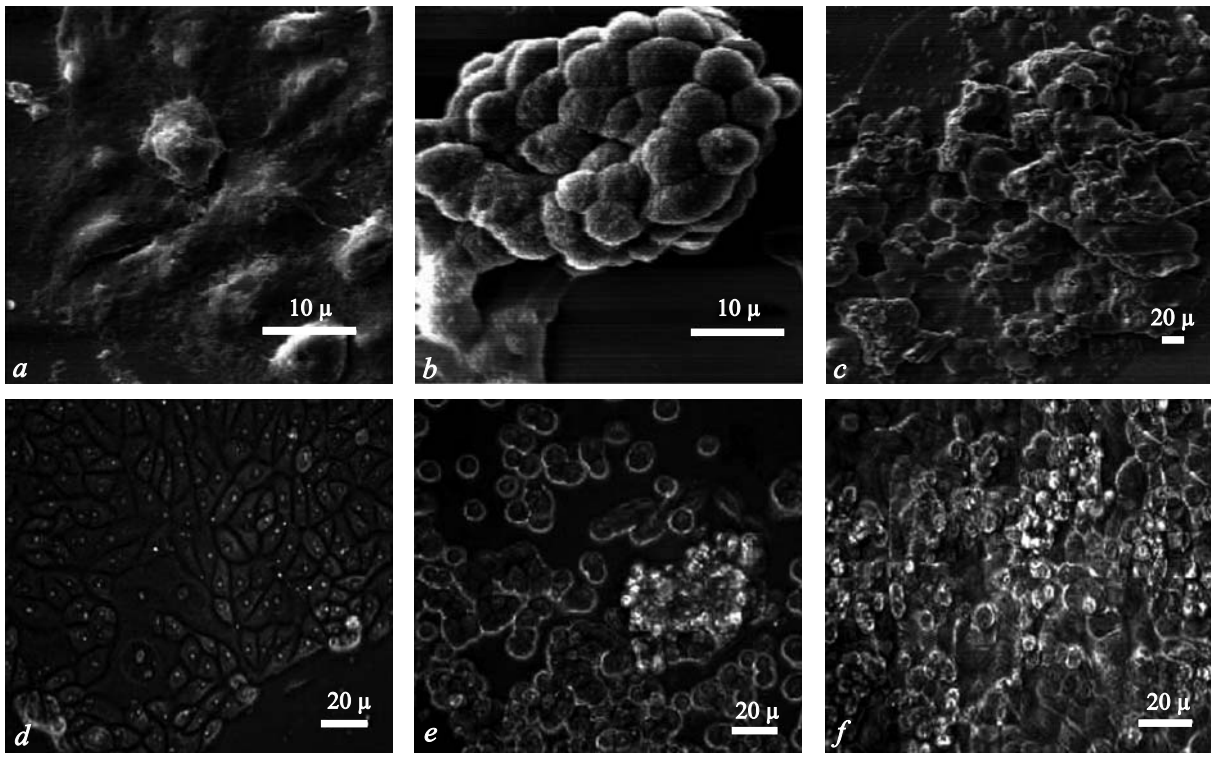

Fig. 4. SPEV cell morphology in 6-day culture.

reactions [4-6,18]. Cascade of molecular rearrangements in actin cytoskeleton structure will determine not only cell–BEFM, but also cell–cell interactions. From the data on SPEV cell morphology (Fig. 3) we can hypothesize that the decrease in cell size (spherical shape on BEFM-1) is associated with contraction of cytoskeleton and changes in integrin receptors and focal contacts and can be considered as a result of potent electrostatic interactions of BEFM surface with cells. Modification of the chitosan matrix by chitin nanofibers is accompanied by changes in voltage, hydrophobicity, and roughness of BEFM-2 surface. Partialy deacetylized chitin is more hydrophobic polymer that chitosan, while chitin nanofibers contain 2-fols less charged groups. These factors, in turn, will determine qualitative and quantitative composition of adsorbed protein molecules on the biopolymer matrix, the character of cell–BEFM interactions, and, consequently, cytoskeleton structure, cell morphology, and proliferation rate. The presence of two cell forms in cultures on BEFM-2 probably reflects heterogenity of the chemical structure of extracellular substrate. Similar results were obtained by other researchers on chitosan matrix modification with gelatin [12]. Electrostatic interactions determined by high degree of deacetylation are limit cell flattening on the surface and induce contraction of actin cytoskeleton leading to a decrease in cell size, which is mediated by changes in adsorbed protein composition [12]. In a previous study [8], the dependence of adhesion and proliferation of keratinocytes and fibroblasts on deacetylation degree of chitosan films (from 2.5 to 47%) has been demonstrated: adhesion and proliferation of keratinocytes increased with reducing the deacetylation rate; no fibroblast proliferation was found on chitosan films.

Thus, physicochemical modification of BEFM surface structure including local changes in its hydrophobicity, roughness, and charge density was the main factor determining cell behavior during *in vitro* culturing. The local decrease in surface charge density on BEFM matrices resulting from introduction of chitin nanofibers with low deacetylation rates led to the appearance of flattened spindle-shaped cells and stimulation of their proliferation in comparison with that on chitosan matrix. Our experiments demonstrated the possibility of creation of a chitosan-based biopolymer film matrix with preset properties for the use in tissue engineering (control of the growth of specific cell types) and regenerative medicine (stimulation of cell adhesion and proliferation in the damaged area).

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