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ONCOLOGY

Effect of Extract from a Transplant for Eyelid Plasty (series ALLOPLANT™) on DNA Synthesis in Cultures Cells

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Extract isolated from a collagen-containing transplant (series ALLOPLANT™), which is used in the treatment of benign and malignant neoplasms of the eyelid, inhibits DNA synthesis in the cell *in vitro*. This effect is nonspecific, reversible, and dose-dependent. The extract is thermostable and resistant to proteolytic enzymes.

Key Words: *eyelid plasty; transplant extract; DNA synthesis; cell culture; ALLOPLANT™*

During the last decade special attention has been focused on the effect of the extracellular matrix (EM) and its components on shape modulation, proliferation and differentiation of various cell types [11,12,14], and cell adhesion to the EM [3]. As is well known EM is a stable complex of macromolecules which can be assigned to 4 classes: collagens, elastins, proteoglycans, and glycoproteins. Proteoglycans and glycoproteins are involved in the regulation of cell proliferation. For example, the proteoglycan heparan sulfate inhibits cell growth; inhibition and stimulation of cell growth have been demonstrated for a number of glycoproteins and glycoconjugates.

Synthesis of artificial analogs of the EM and their use as substrate for cell culturing is one of the major approaches to the elucidation of the role of EM. Such models are convenient tools in the study of the behavior of various cell types [8] and

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of the dependence of proliferative activity and degree of cell differentiation on the structural organization and molecular composition of artificial EM [1,2,6,13].

A transplant for surgical treatment of benign and malignant neoplasms of the eyelid (squamous-cell carcinoma, basal-cell carcinoma, hemangioma, etc.) has been developed and produced at the Russian Center for Eye and Plastic Surgery (Ufa). The transplant is a collagen material isolated from human tissues. It has been approved by the Russian Ministry of Public Health for clinical application and was launched on the market under the trade mark ALLOPLANT™. According to 11-year statistics of use of the transplant in the surgical treatment of oncological diseases at the Center, cancer recurrence was recorded only in 3 patients, i.e., in 1.25% of all patients who underwent the surgery. It should be mentioned that these patients were operated upon after repeated radiation therapy. According to the literature, the recurrence of analogous tumors is 5.6-40%.

It has been suggested that the transplant or some of its components inhibit the growth of tumor cells *in situ*.

The aim of the present study was to examine the effect of extract isolated from the transplant for eyelid plasty (series ALLOPLANT™) on the proliferative activity of some cell lines.

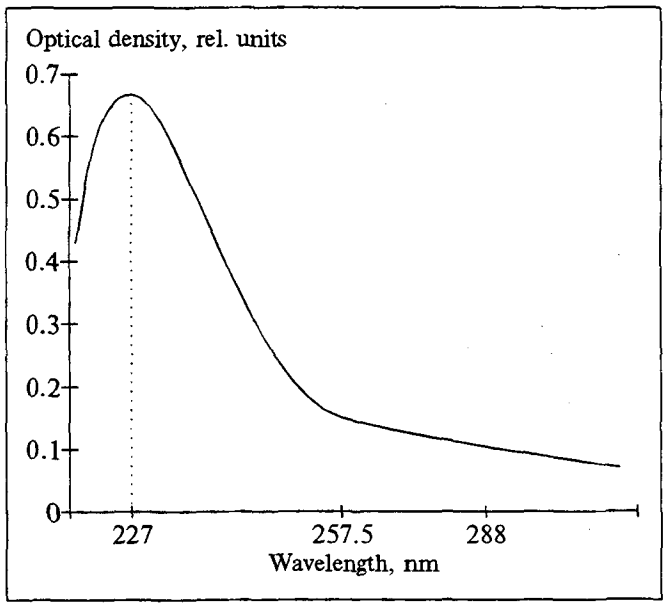

Fig. 1. Ultraviolet absorption spectrum of an aqueous solution of extract isolated from transplant for the eyelid plasty ALLOPLANT™.

MATERIALS AND METHODS

Cell Cultures. CV-1, fibroblastlike heteroploid cells from grivet kidney (Institute of Molecular Genetics, Moscow) cultured in Eagle's MEM medium containing 5% bovine serum and 5% fetal calf serum (Scientific Industrial Association Vektor, Novosibirsk). FLECH 383/13, diploid fibroblasts from human embryonic lung cultured in Eagle's MEM medium containing 5% bovine serum and 5% fetal calf serum. M-HeLa 7886, human cervical carcinoma cells and HEp-2, human larynx carcinoma cells cultured in Eagle's MEM medium containing 5% bovine serum. FLECH 385/13, HEp-2, and M-HeLa 7886 were supplied by the Bank of Cell Lines (Institute of Influenza, St. Petersburg). A-431, human vulva carcinoma cells, and EJ, human bladder carcinoma cells (Division of Surgical Oncology, J. W. Brown Cancer Center, Louisville) were cultured in Dulbecco's modified Eagle medium (Sigma, USA) supplemented with 5% bovine serum (Hazleton Biologic, USA). The following isotopes were used for the analysis of DNA synthesis: [methyl-^{3}H]-thymidine, specific activity 45 Ci/mol (Amersham, Great Britain), [methyl-^{3}H]-thymidine, specific activity 21.5 kCi/mol, and ^{14}C-orotic acid, specific activity 310 mCi/g (Scientific Industrial Association Izotop, St. Petersburg).

The cells were cultured in 24- or 96-well plates (1 ml of medium for 24-well plates and 0.2 ml for 96-well plates). Seeding density was 1.4×10^5 cells/cm^2 for FLECH 385/13, 7×10^4 cells/cm^2 for M-HeLa and HEp-2, and 4×10^4 cells/cm^2 for A-431 and EJ. The cultures were incubated at 37°C in 95% air/5% CO_2 until 50% confluence was reached (usually it took 24-48 hours). The culture medium was then replaced with fresh medium containing the extracts.

TABLE 1. Effect of Extract (500 μg/ml) from the ALLOPLANT™ Transplant on DNA Synthesis in Cultured Cells ($M\pm m$, $p<0.05$)

Cell culture	DNA synthesis, dpm		Degree of inhibition, %
	control	experiment	
CV−1	674±42	209±20	69
FLECH	5275±182	2879±135	45
HeLa	16266±278	10403±865	36
HEp−2	2139±160	1349±96	37

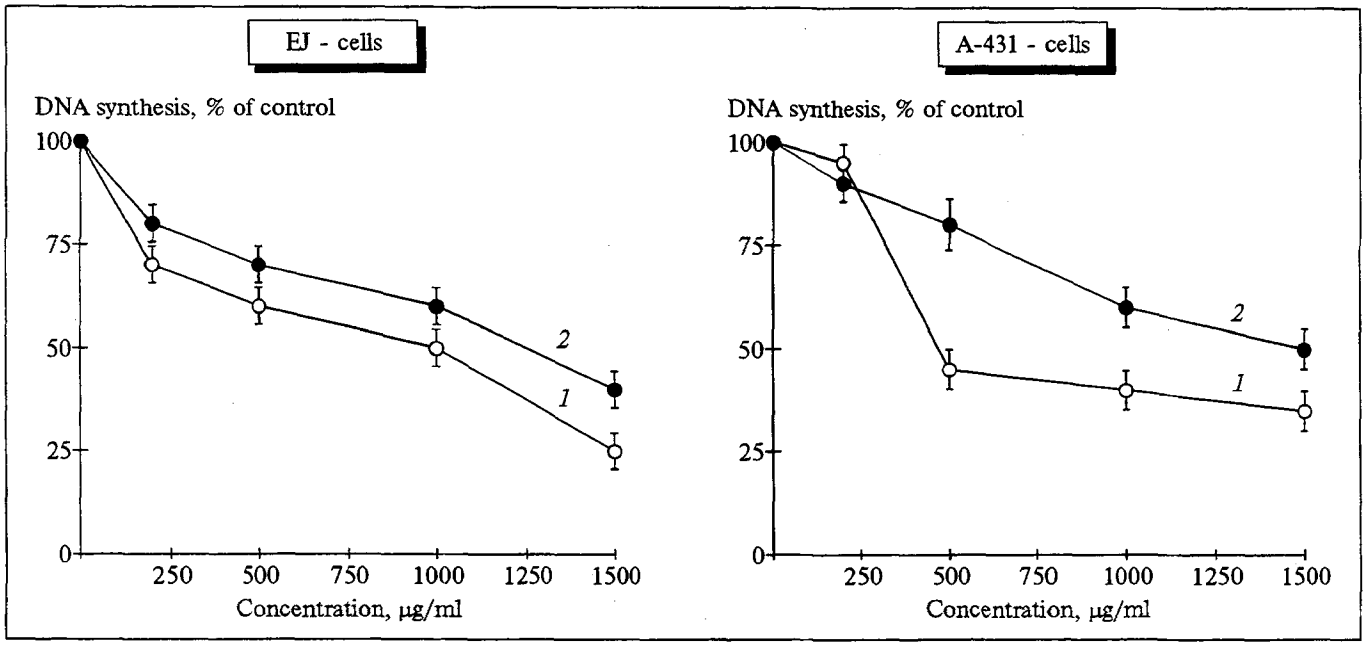

Fig. 2. Dose–dependent inhibition of DNA synthesis in cultured EJ and A–431 cells. *1*) changes in DNA synthesis caused by the ALLOPLAN™ extract; *2*) changes in DNA synthesis caused by extract from hyaline cartilage.

In some experiments the cells were incubated with the extract for 24 h, and then the culture medium was replaced with fresh medium (control) or medium containing the same concentrations of the extract (experiment). The cells were labeled with [methyl-^{3}H]-thymidine (1 μCi/ml) or ^{14}C-orotic acid (0.5 μCi/ml). The label incorporation in DNA and RNA was determined after 24 h, as described [9].

In other experiments the cells were incubated with the extract for 24 hours in the presence of labeled thymidine.

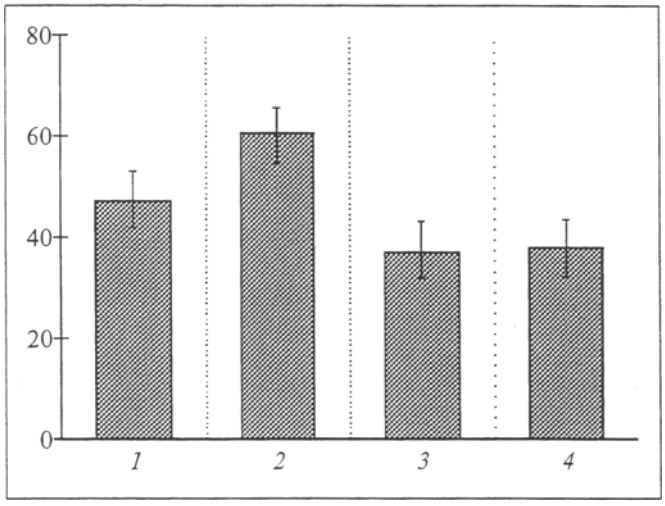

Fig. 3. Inhibition (%) of DNA synthesis in cultured A–431 cells by extract from the ALLOPLANT™ transplant (500 μg/ml) and extract from hyaline cartilage (500 μg/ml) heated to 95°C for 30 min. *1*) extract from the ALLOPLANT™ transplant; *2*) the same extract after heat treatment; *3*) extract from hyaline cartilage; *4*) the same extract after heat treatment.

The results were presented as the total radioactivity (decays per minute, dpm) of the material obtained from each sample. The significance of differences between the mean values of the label incorporation in the nucleic acids of the control and experimental groups was evaluated using Student's *t* test.

Two approaches were utilized to estimate the resistance of the extract to high temperature. The first one consisted in the following: the extract was weighed, dissolved in water, kept for 30 min at 95°C, cooled, and lyophilized in a Savant vacuum concentrator (USA). The preparation was dissolved in culture medium and used in the experiment. In the second approach the extract was dissolved in Hank's balanced salts and kept at 95°C for 30 min. The solution was then added to the culture medium to obtain the required concentration. Culture medium with an equal content of heated Hank's salts was used as control.

For estimation of the resistance of the extract to proteolytic enzymes, the extract was dissolved in Eagle's MEM medium and incubated with a profezyme suspension at 37°C for 1.5 h with periodical shaking. The profezyme preparation was the enzyme protosubtilin from *Bacillus subtilis* and immobilized on AE-Cellulose (Vektor, Novosibirsk). After centrifugation (2 min, 5000 g), the supernatant was collected and used in the experiment. Heat-inactivated (95°C, 30 min) profezyme preparation was used as a control.

Hexoses were determined with the anthrone reagent, using glucose as the standard. The reac-

tion for uronic acids was carried out by the Dishet method, using heparin as the standard. The protein content was determined after Lowry with bovine serum albumin as the standard.

RESULTS

Preliminary biochemical analysis of the extract isolated from the transplant for the eyelid plasty (series ALLOPLANT™) showed that this extract contains 21% protein. The reaction for hexoses was negative. Glycosaminoglycans containing uronic acid residues (68%) were the major constituent of the preparation.

The analysis of the ultraviolet spectrum (Fig. 1) showed that the preparation contains no nucleic acids (no peak at 257 nm). The absence of the 280-nm peak indicates also that the preparation contain no aromatic amino acids.

It was shown that the extract inhibits DNA synthesis in normal and tumor cells, the inhibition being statistically significant (Table 1).

The preparation exerted no cytotoxic effect: after incubation with the extract, the cells remained spread, had the characteristic glitter, contained no pathological vacuoles, and displayed no cytoplasm granulation. However, high dose of the preparation (1500 μg/ml) caused cell detachment and death. It was shown that inhibition of DNA synthesis is dose-dependent: high doses of the preparation produced a strong inhibitory effect and lower doses had a weak effect. A comparison of the dose-dependent inhibition of DNA synthesis by the extracts isolated from the ALLOPLANT™ transplant or hyaline cartilage on EJ (Fig. 2, *a*) and A-431 (Fig. 2, *b*) showed that both preparations act in a similar manner.

A simultaneous use of labeled precursors of RNA and DNA in the experiments with CV-1 cells enabled us to discover that at a very strong inhibition (68%) of ^{3}H-thymidine incorporation the extract produced a weak effect (14%) on the incorporation of ^{14}C-orotic acid in RNA (Table 2).

The inhibitory effect on DNA synthesis is not abolished by the addition of the extract heated to 95°C for 30 min (Fig. 3) or treated with a proteolytic enzyme (Fig. 4).

The experiment with A-431 cells was performed to study the reversibility of the effect of

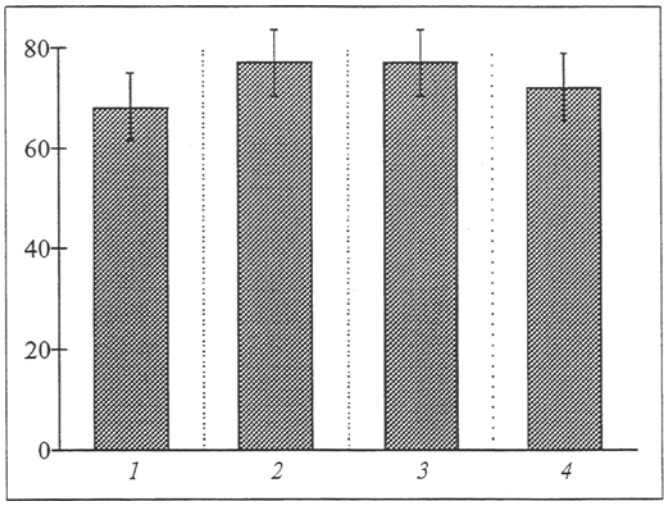

Fig. 4. Inhibition (%) of DNA synthesis in cultured CV−1 cells by extract from the ALLOPLANT™ transplant (500 μg/ml) treated with a proteolytic enzyme (profenzyme). *1*) extract from the ALLOPLANT™ transplant; *2*) the same extract after heat treatment (95°C, 30 min); *3*) extract from the ALLOPLANT™ transplant treated with inactivated profenzyme; *4*) extract from the ALLOPLANT™ transplant treated with the profenzyme.

the extract on DNA synthesis. The preparation was added to the culture medium at a concentration of 500 μg/ml. After a 24-h incubation, the medium was removed from two rows of the plate. Fresh medium was added to the wells of one of these rows, and medium containing extract was added to the wells of the other. The medium was not replaced in the third row. The cells were incubated in the presence of ^{3}H-thymidine (1 μCi/ml) for 24 hours, and the label incorporation was evaluated. The highest degree of inhibition (70%) was recorded when the preparation was added during 48 h of culturing. The inhibition was lower (66%) in the row where the medium with the preparation was not changed, and it was considerably lower in the row where the medium was replaced with the fresh (Table 3). Thus, the extract from the transplant for the eyelid plasty retains its activity and has an inhibitory effect on cell proliferation during 48 hours. The mechanisms of DNA synthesis start operating once the extract had been removed from the culture medium.

The results obtained led to the preliminary conclusion that the extract contains considerable amounts of glycosaminoglycans with uronic acid residues. As is well known, hyaluronic acid, chondroitin sulfates, dermatan sulfate, and heparan sul-

TABLE 2. Effect of Extract (500 μg/ml) from the ALLOPLANT™ Transplant on DNA Synthesis in CV−1 Cells ($M \pm m$, $p<0.05$)

Experimental conditions	DNA synthesis, dpm	Inhibition of DNA synthesis, %	RNA synthesis, dpm	Inhibition of RNA synthesis, %
Control	233±19	–	205±9	–
Experiment	71±2	68%	158±9	23%

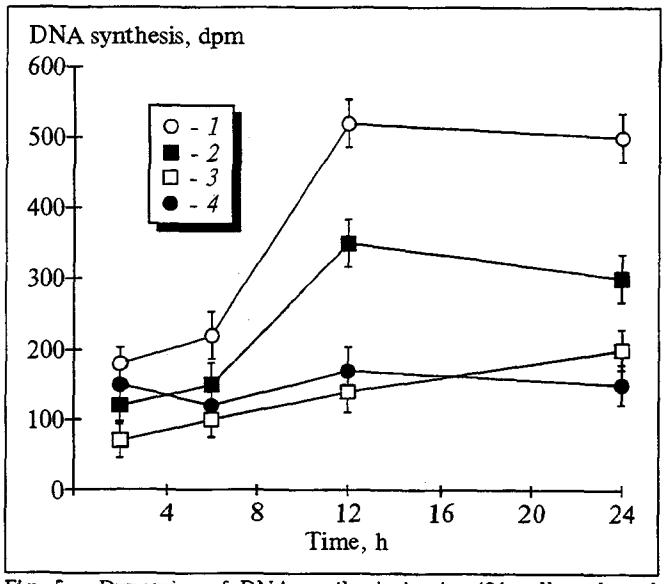

Fig. 5. Dynamics of DNA synthesis in A−431 cells cultured in the presence of heparin, extract from the ALLOPLANT™ transplant, and extract from hyaline cartilage. *1*) control; *2*) extract from the ALLOPLANT™ transplant (500 μg/ml); *3*) extract from hyaline cartilage (500 μg/ml); *4*) heparin (100 μg/ml).

fate are uronic acids. The last three compounds generally function as proteoglycans, which are indispensable components of EM of different origin [3].

The matrix determines cell proliferation, adhesion, and morphology. It also plays an important role in selective filtration of solutions.

The extract prepared from the transplant acts on the cells as a physiological regulator of proliferation, but not as a toxin, i.e., cells remain viable after being exposed to the extract. This is confirmed by the reversibility of the effect of the extract.

A study of the dynamics of the extract's effect on DNA synthesis in cultured cells (Fig. 5) showed a similarity between the action of the extract and heparin, a strong inhibitor of cell proliferation. A similar effect was demonstrated for the EM component heparan sulfate proteoglycan, which contains side-chain glycosaminoglycans with a molecular weight of 15-18 kD. It has been reported that heparan sulfates display a growth-inhibiting activity; the ability to inhibit or stimulate cell growth has also been demonstrated for glycoproteins and glycoconjugates.

The data on the dose-dependence of the effect of the preparation agree with the results obtained in an earlier study of an antitumor factor isolated from cartilage. Therefore, further investigation of the biochemical nature of the active ingredient of the preparation is important for us.

So far, it is difficult to identify this ingredient. Presumably, this is a polymeric material that cannot pass through the pores during dialysis, it is hydrophilic, judging from its readiness to dissolve in water-salt solutions. The compound is heat-stable: the extract remains active after being frozen to -20°C and thawed, or being heated to 95°C for 30 min. The fact that the preparation remains active after treatment with a proteolytic enzyme indicates that the molecule 1) is not a pure protein, 2) is rather a highly glycosylated glycoprotein or proteoglycan, in which the binding sites potentially sensitive to the protease are sterically protected, or 3) is a protein containing no sites sensitive to the protease chosen in this study.

A compound which is an integral component of the EM of human connective tissues can be proposed as a candidate for the active ingredient.

In the present study we have examined the effect of an extract from the transplant for eyelid plasty (series ALLOPLANT™) on cell proliferation *in vitro*. The collagen-based transplant is very interesting for such studies. Collagen substrates are known to affect cell growth and to modulate various aspects of cell behavior, including adhesion and spreading. In addition, proliferative activity and the capacity for differentiation are determined by the structure of the collagen substrate. The behavior of cells depends on the culturing conditions under which they are grown: on a collagen, on collagen sponge, or in a three-dimensional collagen gel [4,10,15]. This fact is important for planning

TABLE 3. Effect of the ALLOPLANT™ Transplant on DNA Synthesis in Cultured A−431 Cells under Different Incubation Conditions ($M \pm m$)

Incubation conditions	DNA synthesis, dpm		Inhibition of DNA synthesis, %	p
	control	experiment		
Extract added at a concentration of 500 μg/ml and changed after 24−h incubation	289±51	86±5	70	<0.05
Extract added at a concentration of 500 μg/ml and incubated with cells for 48 h	279±78	94±7	66	<0.01
Extract added at a concentration of 500 μg/ml After 24−h incubation fresh medium was added	290±51	142±13	51	>0.05

further investigations, since the transplant for eyelid plasty ALLOPLANT™ is a collagenous material with the characteristic architectonics.

There is also the possibility that the compounds which forming from the matrix component during technological processing of the transplant elicit a nonspecific inhibitory effect.

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Determination of the Biological Activity of Chalone-Containing Preparation from Ehrlich Ascitic Carcinoma and Its Fractions Obtained by High-Performance Liquid Chromatography in a Cell Culture Derived from This Tumor

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It is shown that cultured Ehrlich ascitic carcinoma cells are a convenient test system for the investigation of the effects of various factors on DNA synthesis in the cells of this tumor. The application of this system markedly facilitates fractionation of a chalone-containing preparation, the purpose of this fractionation being the isolation of components affecting specific phases of the mitotic cycle.

Key Words: *Ehrlich ascitic carcinoma; high-performance liquid chromatography; chalone; DNA synthesis*

Estimation of the biological activity of chalone-containing preparations (CCP) is an important task in the purification of CCP from various tissues. Each step of CCP separation requires the determination of chalone activity in separate fractions. At the present time, a variety of methods are used to determine the biological activity of CCP both *in*

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