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ORIGINAL CONTRIBUTIONS

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INHIBITION OF MALIGNANT POTENTIAL AND EXPRESSION OF PROTEINS ASSOCIATED WITH EPITHELIAL-MESENCHYMAL TRANSITION IN LEWIS LUNG CARCINOMA CELLS TRANSDUCED WITH MURINE *ifn-β* GENE IN RECOMBINANT BACULOVIRUS

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Aim: To analyze biological characteristics, malignant potential and expression of proteins associated with epithelial-mesenchymal transition in murine lung carcinoma cells transduced with interferon-beta (*ifn-β*) gene in baculovirus vector. *Materials and Methods:* The study was performed on Lewis lung carcinoma (LL) cells transduced with *ifn-β* gene in recombinant baculovirus vector. Biological characteristics of the LL cells were studied with the use of standard cell culture methods, cytogenetic and immunocytochemical assays. *Results*: Recombinant baculovirus-mediated transduction of LL cells with *ifn-β* gene resulted in significant decrease of cell growth rate and density both in complete and serum-free medium. Also, LL cells transduction with *ifn-β* gene significantly inhibited cell migration *in vitro*. Transduction of LL cells by baculovirus vector with or without *ifn-β* gene caused significant genotoxic effect in these cells. Furthermore, *ifn-β* gene transfer to lung carcinoma cells resulted in significant increase of nuclear expression of $p19^{ARF}$ ($p < 0.01$), $p21^{WAF1}$ ($p < 0.001$), cytoplasmic expression of E-cadherin ($p < 0.005$) and inhibition of transcription factors of epithelial-mesenchymal transition (EMT) Twist ($p < 0.005$) and Slug ($p < 0.001$) expression. *Conclusions*: Transduction with *ifn-β* gene of LL cells in recombinant baculovirus resulted in acquirement of less malignant phenotype *in vitro* and suppressed expression of proteins associated with EMT.

Key Words: interferon-beta, lung cancer, malignancy, recombinant baculovirus, cytogenetic assay, epithelial-mesenchymal transition.

It is well-known that type I interferons (IFNs), including interferon-beta (IFN-β) exert anti-tumor effects via suppression of tumor cell proliferation and stimulation of apoptosis, affecting host immune system, etc. [1–3]. Especially important, recently IFN-β has been shown to be capable to inhibit the process of epithelial-mesenchymal transition (EMT) [4–7].

Also, IFNs exert immunoregulatory effects toward antibody production, activation of T cell killing, macrophage function and delayed-type hypersensitivity [8]. Moreover, IFN-β affects not only the tumor cells but also the cell elements of tumor microenvironment providing antiangiogenic effect [9].

Delivery of type I IFN proteins into tumors via intravenous or intramuscular routes has been explored in the therapy of many cancer types. Unfortunately, side effects of IFN used at maximally tolerated dose, and its short half-life (< 60 min) makes it difficult to achieve sustained therapeutic levels of IFN in the tumors [2, 9]. The use of pegylated IFN increases the stability of the protein, but does not provide the desired local therapeutic levels.

To counteract these limitations, a number of investigators have shown that *in vivo* gene delivery of the *ifn-α* or *ifn-β* gene using gene transfer methods such as plasmids or various viral vectors, could be effective in tumor models of metastatic cancers (lung, breast, bladder, pancreatic), renal cell carcinoma, glioma, and liver metastases of colorectal cancer [10–12]. In some studies *ifn-β* gene therapy in lung cancer has been used [5, 7, 10, 13–15]. Previously, using the model of Lewis lung carcinoma (LL) cells we have demonstrated suppression of proliferation, tumorigenicity and metastasis of tumor cells *in vivo* after their transduction with *ifn-β* gene cloned in baculovirus vector [7]. However, in this research we have not study *in vitro* the main biological and cytogenetic characteristics of LL cells transduced with *ifn-β* gene (their morphology, growth rate and density, migration activity, apoptosis, polyploidy, cytogenetic abnormalities). Also, the aim of present work was to analyze whether the production of endogenous IFN-β in transduced LL cells altered the expression of proteins associated with cell cycle regulation, invasive and migration potential of these cells *in vitro* and EMT of lung cancer cells.

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Abbreviations used: ACCC – asynchronous cleavage of centromeric regions of chromosomes; BV – baculovirus without *ifn-β* gene; BV/IFN – recombinant baculovirus vector with *ifn-β* gene; CA – chromosome aberrations; CMN – cells with micronuclei; DMEM – Dulbecco's Modified Eagle's medium; EMT – epithelial-mesenchymal transition; IFN-β – interferon-beta; *ifn-β* – interferon-beta (gene); IFNs – type I interferons; LL – Lewis lung carcinoma; MOI – multiplicity of infection; NCS – newborn calf serum; PBS – phosphate buffered saline; Protr – protrusions; Rb – Robertsonian translocations.

MATERIALS AND METHODS

Cell line and recombinant virus. LL cells (LL cell line) [16] were obtained from the Bank of cell lines from human and animal tissues of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine. The cells were routinely maintained in complete Dulbecco Modified Eagles Medium

(DMEM) (with 4 mmol/l L-glutamine) (BioWest, France) supplemented with 10% newborn calf serum (NCS) (BioWest, France) and 40 mg/ml gentamicin (Sigma, USA) in a CO_2-incubator at 37 °C.

The recombinant baculovirus vectors were prepared as described [17, 18]. The recombinant baculoviruses are based on the *Autographa californica* multiple nucleopolyhedrovirus. Recombinant vector, containing murine *ifn-β* gene under the control of strong CAG promoter cassette (BV/IFN) was used as the IFN-β expressing vehicle. Vector without the *ifn-β* gene (BV) used as a control.

Also, LL cells were cultured with exogenous IFN-β (Sigma, USA, Cat. № 19032) at concentrations 200 IU/ml (this concentration is close to that of IFN endogenously produced by BV/IFN transduced LL cells).

Transduction method. LL cells in RPMI-1640 medium (BioWest, France) were mixed with baculovirus (with or without *ifn-β* gene at multiplicity of infection (MOI) 100) in phosphate buffered saline (PBS) in a ratio of 1:1 and incubated at 25 °C for 2 h. Then LL cells were cultivated in DMEM supplemented with 10% NCS and 40 mg/ml gentamicin for 1–12 days. The control LL cells underwent similar procedure where PBS without BV was used.

Analysis of growth kinetic of tumor cells. LL cells ($1 \cdot 10^4$ cells/ml) were seeded in 24-wells plates (TPP, Italy) in 1 ml of complete DMEM and incubated for 12 days. Every 24 h the cells were detached with EDTA solution (BioTestMed, Ukraine) from three wells at each time point, stained with trypan blue solution (HyClone, USA) and counted in hemocytometer. All experiments were repeated twice.

Growth of tumor cells in serum-free medium. LL cells ($1 \cdot 10^4$ cells/ml) were seeded in 24-wells plates in 1 ml of complete DMEM, and incubated for 24 h. Thereafter the medium was replaced with fresh DMEM without serum and were further cultured for 12 days. Every day the cells were detached with EDTA solution from three wells at each time point, stained with trypan blue solution and counted in hemocytometer. All experiments were repeated twice.

Scratch assay. LL cells ($5 \cdot 10^4$/ml) were seeded in 12-well plate (TPP, Italy) in DMEM in the presence of 10% NCS, 40 μg/ml gentamicin and incubated until cells in most study groups reach 100% confluence. We used a p200 pipette tip to create a scratch on the cell monolayer surface. After that the medium in each well was replaced with fresh complete DMEM to remove debris and to smooth the edge of the scratch. Process of LL cell migration in the "scratch" was observed with phase contrast microscope and photographed in several time points: 0 h, 24 h and 48 h. The images were analyzed and the incubation period required to restore the scratch was determined [19].

Cytogenetic analysis. 72 h after transduction LL cells were detached with EDTA solution and incubated for 40 min in hypotonic solution of KCl (0.54%) (Reahim, Ukraine) at 37 °C. After that the cells were fixed with methanol (Reahim, Ukraine) and acetic acid (Chemlaborreactiv, Ukraine) in a ratio of 3:1. The procedure of fixing was performed in triplicate. Cell suspension in the fixing solution was dripped on the cold wet glass slides and dried. Samples were stained with Giemsa stain (Merck, Germany). The cytogenetic preparations were analyzed using binocular Axiostar Plus microscope (Carl Zeiss, Germany) at the magnification × 100 and photographed using camera Canon PowerShot G5 (UK). The following characteristics were studied: number of mitoses, cells with micronuclei (CMN), apoptotic cells and cells with nuclear protrusions (Protr). These indicators were counted per 1000 cells, and the results are expressed in per mille (‰). Mitotic regime and pathology of mitoses were counted per 300 of mitoses and expressed as a percentage (%). In the mitoses the frequency of cells with chromosome aberrations (CA), asynchronous cleavage of centromeric regions of chromosomes (ACCC), Robertsonian translocations (Rb) and association between chromosomes by type of Robertsonian translocations, modal class of chromosomes by the number of acrocentrics and modal class of chromosomes based on association between chromosomes as separate chromosomes were analyzed [20].

Immunocytochemical assay. Phenotype changes of LL cells after their transduction with recombinant baculoviruses were determined by counting the cells expressing cell cycle regulation proteins $p19^{ARF}$ and $p21^{WAF1}$, transcription factors Twist and Slug and adhesion molecules E-cadherin, N-cadherin. Just after incubation of LL cells with viruses, they were placed on the coverslip and 72 h later the analysis was made by the standard method [21] with the use of monoclonal antibodies against $p21^{WAF1}$ (NeoMarkers, USA), $p19^{ARF}$ (Thermo Scientific, USA), Twist and Slug (Gen Tex, USA), E-cadherin (Thermo Scientific NeoMarkers, USA), N-cadherin (BioLegend, USA). Ultra Vision LPValue Detection system (Thermo Scientific, USA) was used to visualize the reaction of antigen/antibody. DAB Quanto (3,3-diaminobenzydyn) (Thermo Scientific, USA) was used as a chromogen. Stained preparations were analyzed using binocular Axiostar Plus microscope and immersion oil at the magnification × 100 and photographed using camera Canon PowerShot G5.

Expression of mentioned proteins was evaluated semiquantitavely using H-score system accounting the number and intensity of the stained cells. The percentage of the stained cells was multiplied by score number corresponding to the staining intensity (0 = none, 1 = weak, 2 = moderate, 3 = strong). The resulting score ranged from 0 (no stained cells) to 300 (diffuse intense staining of cells), and H-score was calculated as (percentage of cells stained at intensity 1×1) + (percentage of cells stained at intensity 2×2) + (percentage of cells stained at intensity 3×3) [22].

Statistical analysis. Statistical data processing was performed by the Student t-test. Results are presented as M ± SD.

RESULTS

The growth kinetics of LL cells in complete and serum-free medium after their transduction with ifn-β gene in recombinant baculovirus. We have study the growth kinetics of LL cells after their transduction with *ifn-β* gene in BV (LL/BV/IFN), without inserted gene (LL/BV), LL cells treated with 200 IU/ml of exogenous mouse IFN-β (LL + IFN-β) as well as untreated LL cells (LL control). The growth kinetics of mentioned above cells analyzed within 12 days is presented in Fig. 1, *a*.

The LL/BV/IFN cells demonstrated the slowing the exponential growth rate as compared with LL/BV cells, LL + IFN-β cells and especially with untreated LL control cells (9 days *vs* 8 days for LL/BV cells, LL + IFN-β cells and 7 days for control cells). The density of cell growth was dramatically inhibited in the LL/BV/IFN cells ($1.4 \cdot 10^5/cm^2$) compared to LL control cells ($6.3 \cdot 10^5/cm^2$) and $5.1 \cdot 10^5/cm^2$, $5 \cdot 10^5/cm^2$ for LL/BV, LL + IFN-β cells, respectively (Fig. 1, *a*).

Our data showed that transduction of LL cells with BV/IFN leads to the most significant inhibition the ability of these cells to grow in serum-free medium compared to other experimental cell groups. In this case, the density of cell growth in serum-free medium was the lowest in LL/BV/IFN cells ($0.4 \cdot 10^5/cm^2$). In control LL cells this indicator was equal to $2.8 \cdot 10^5/cm^2$, in LL/BV and LL + IFN-β cells amounted $1.4 \cdot 10^5/cm^2$ and $1.2 \cdot 10^5/cm^2$, respectively (Fig. 1, *b*).

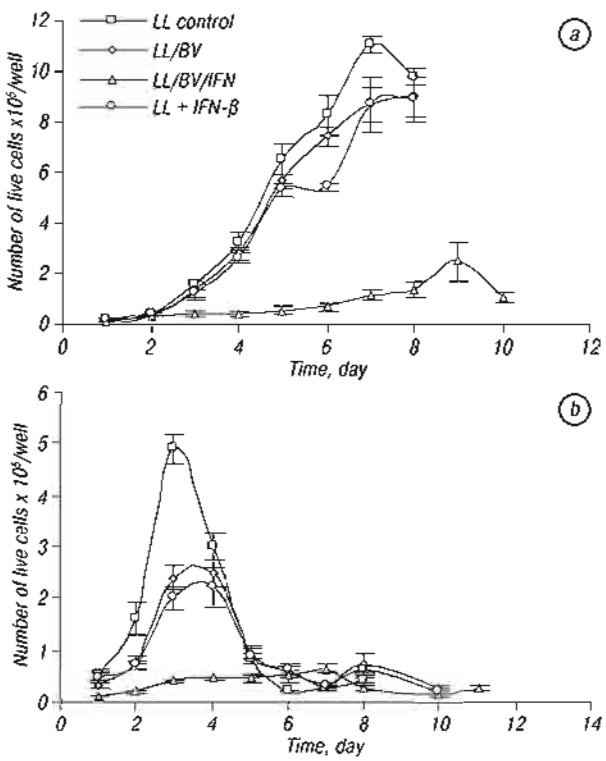

Fig. 1. Features of the kinetics of LL cell growth after their transduction with *ifn-β* gene in baculovirus vector (*a*). The growth of LL cells in serum-free medium after their transduction with *ifn-β* gene in baculovirus vector (*b*)

Analysis of LL cells migration. One of the simplest methods of cell migration analysis *in vitro* is scratch assay. Our studies showed that transduction of recombinant *ifn-β* gene in the LL cells causes suppression of their migratory activity. Monolayers of control LL and LL/BV cells are restored during 48 h after the damage (Fig. 2, *a*, *b*), while cells transduced with *ifn-β* gene cannot restored monolayer even after 120 h of cultivation (Fig. 2, *c*). LL cells treated with exogenous IFN-β completely restore the monolayer after 60 h (Fig. 2, *d*).

Cytogenetic characteristics of LL cells transduced with ifn-β gene in baculovirus vector. Significant changes of biological properties of BV/IFN transduced LL cells were associated with the change of their cytogenetic characteristics. The experiments shown that transduction of LL cells with baculovirus without inserted genes and their short-term treatment with exogenous IFN-β caused significant increase of the level of CA in metaphases and the frequency of appearance of nuclear Protr by 2 times compared to control LL cells (Table 1, 2).

Whereas, in LL cells transduced with *ifn-β* gene we have observed not only twofold increase of CA level (see Table 2), but also 2-fold increase of the number of CMN, significant decrease of the number of mitoses: from 29.7 ± 2 in control LL cells to 16 ± 2.6 in LL/BV/IFN cells (Table 1). Furthermore, additional chromosomal fusion type like Rb, and an increased frequency of ACCC were more often registered in these cells (see Table 2).

Table 1. Changes of cytogenetic characteristics of LL cells after their transduction with *ifn-β* gene in recombinant baculovirus vector

Cytogenetic characteristics	LL control	LL/BV	LL + IFN-β	LL/BV/IFN
CMN, ‰	7.3 ± 2.3	6.0 ± 1.0	7.3 ± 0.6	16.3 ± 2.0**
Mitosis, ‰	29.7 ± 2.0	36.3 ± 8.9	26.3 ± 14.5	16.0 ± 2.6***
Apoptosis, ‰	0.7 ± 1.1	0.3 ± 0.6	1.3 ± 0.6	6.3 ± 2.0*
Protr, ‰	7.7 ± 2.0	14.3 ± 2.5*	14.0 ± 2.6*	9.3 ± 2.5

Note: difference *vs* control LL cells is statistically significant; *$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$.

Table 2. Karyological characteristics of LL cell transduced with *ifn-β* gene in recombinant baculovirus

Metaphases with	LL control	LL/BV	LL + IFN-β	LL/BV/IFN
CA, %	7.8 ± 1.5	18 ± 2.0**	18 ± 2.0**	16 ± 1.2**
ACCC, %	2.6 ± 0.6	2 ± 1	3 ± 0.5	5.8 ± 1.0*
Modal class with Rb (number of chromosomes)	57–61	57–60	57–60	59–61
Cells with modal class, %	74	55	63	46

Note: difference *vs* control LL cells is statistically significant; *$p \leqslant 0.05$, **$p \leqslant 0.001$.

The transduction of LL cells with BV/IFN caused most expressed genotoxic effect with increased apoptosis, polyploidy and endoreduplication rate as compared to other groups (Fig. 3, *c*–*d*). Moreover, the number of annular metaphases was also higher in these samples than in all others. Delay of cell cycle in anaphase and telophase was observed in LL/BV/IFN cells. It is necessary to point that IFN-β used alone caused chromatid bridge. Also, in LL cells transduced with *ifn-β* gene we have observed the decrease of the modal class of chromosomes (Table 3) and increased frequency of chromosomal fusion type like Rb: 9 Rb in LL/BV/IFN cells *vs* 6 Rb in control LL cells and 7 Rb in LL/BV and LL + IFN-β cells.

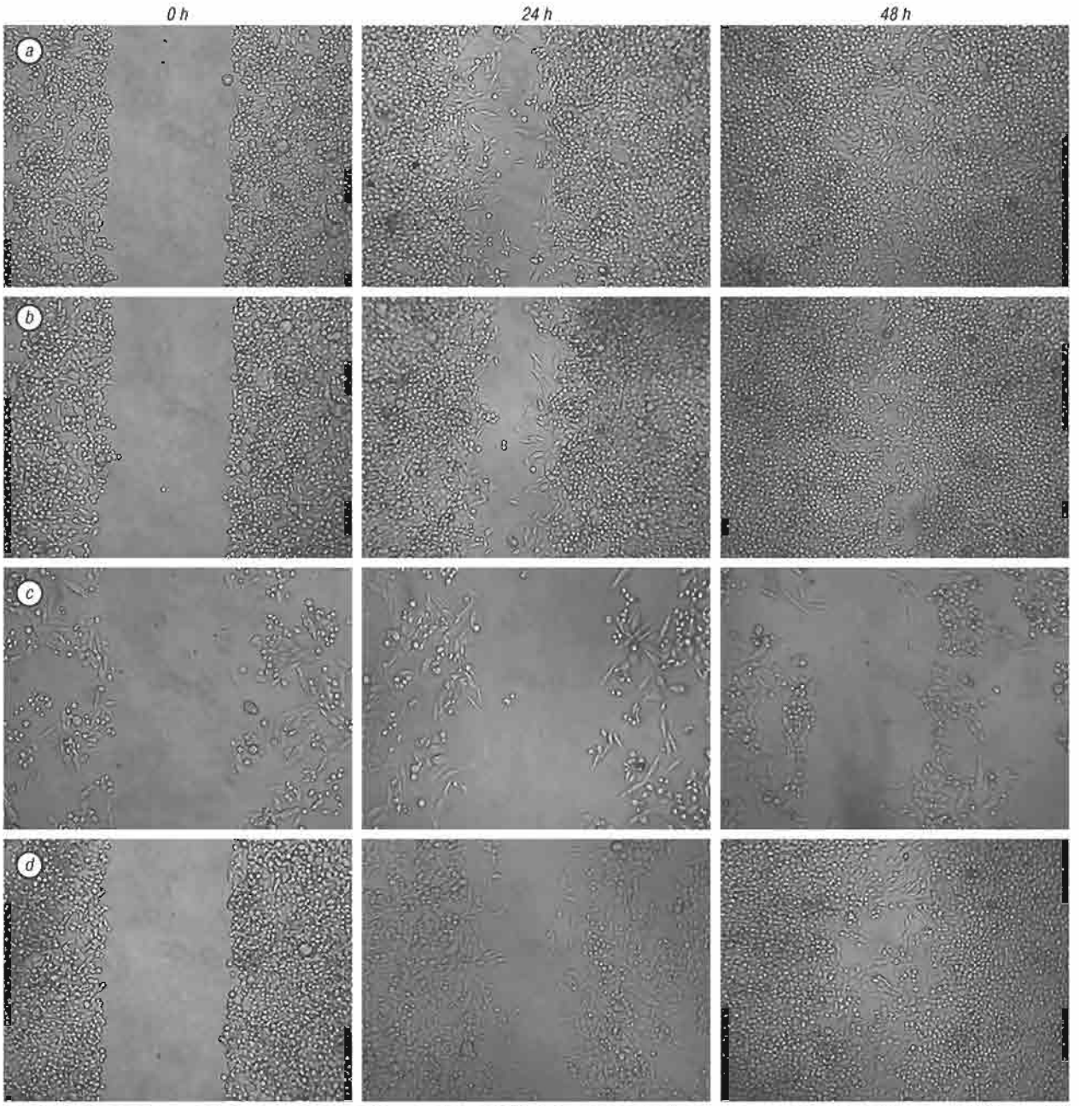

Fig. 2. Analysis of mouse lung carcinoma cells migration activity by *in vitro* scratch assay: *a* — control LL cells; *b* — LL/BV; *c* — LL/BV/IFN; *d* — LL + exogenous IFN-β

Table 3. Mitotic abnormalities in LL cells after their transduction with *ifn-β* gene in recombinant baculovirus

Pathology of mitosis	LL control	LL/BV	LL + IFN-β	LL/BV/IFN
Annular metaphase, %	3.5 ± 1.0	5.7 ± 1.6	3.9 ± 1.3	9.2 ± 1.5**
Backlog of 1 chromosome, %	1.1 ± 0.4	1.5 ± 0.5	1.4 ± 0.3	1.6 ± 0.5
Backlog the group of chromosomes, %	1.7 ± 0.5	1.4 ± 0.7	1.3 ± 0.6	1.3 ± 0.7
Chromatid bridge, %	0	0	0.4 ± 0.1**	1.2 ± 0.3**
Dispersion of chromosomes, %	2.3 ± 1.0	2.1 ± 0.6	2.9 ± 0.8	2.2 ± 0.5
Polyploidy, %	2.4 ± 0.7	1.0 ± 0.4	2.3 ± 0.5	4.0 ± 0.4*
Endoreduplication, %	0.2 ± 0.2	0.6 ± 0.2	0.4 ± 0.3	3.0 ± 0.5**
The total number of pathology, %	11.2	12.3	12.6	22.5

Note: difference *vs* control LL cells is statistically significant; *$p \leq 0.05$, **$p \leq 0.001$.

Changes in the expression of proteins associated with EMT in LL cells after their transduction with BV/IFN. After analysis of all the results we hypothesized that transduction of LL cells with *ifn-β* gene in BV vector can lead to changes in expression of several proteins involved in cell cycle regulation. Also, the transduction of carcinoma cells with *ifn-β* gene affects the expression of the EMT transcription factors Twist and Slug, as well as adhesion molecules — E-cadherin and N-cadherin, associated with invasive and migration potential of tumor cells. The results of these studies can explain some mechanisms that ensure changes of biological properties of tumor cells after their transduction with BV/IFN. Transduction of LL cells with BV/IFN was accompanied by a significant reduction (after 72 h) of cytoplasmic expression of $p19^{ARF}$ protein (250 ± 27 points in control *vs* 50 ± 5 points in LL/BV/IFN cells) and the number of expressing cells by 55%. In addition, the synthesis and production of recombinant IFN-β in LL/BV/IFN cells was accompanied by a significant increase of nuclear expression of $p19^{ARF}$ (Table 4, Fig. 4).

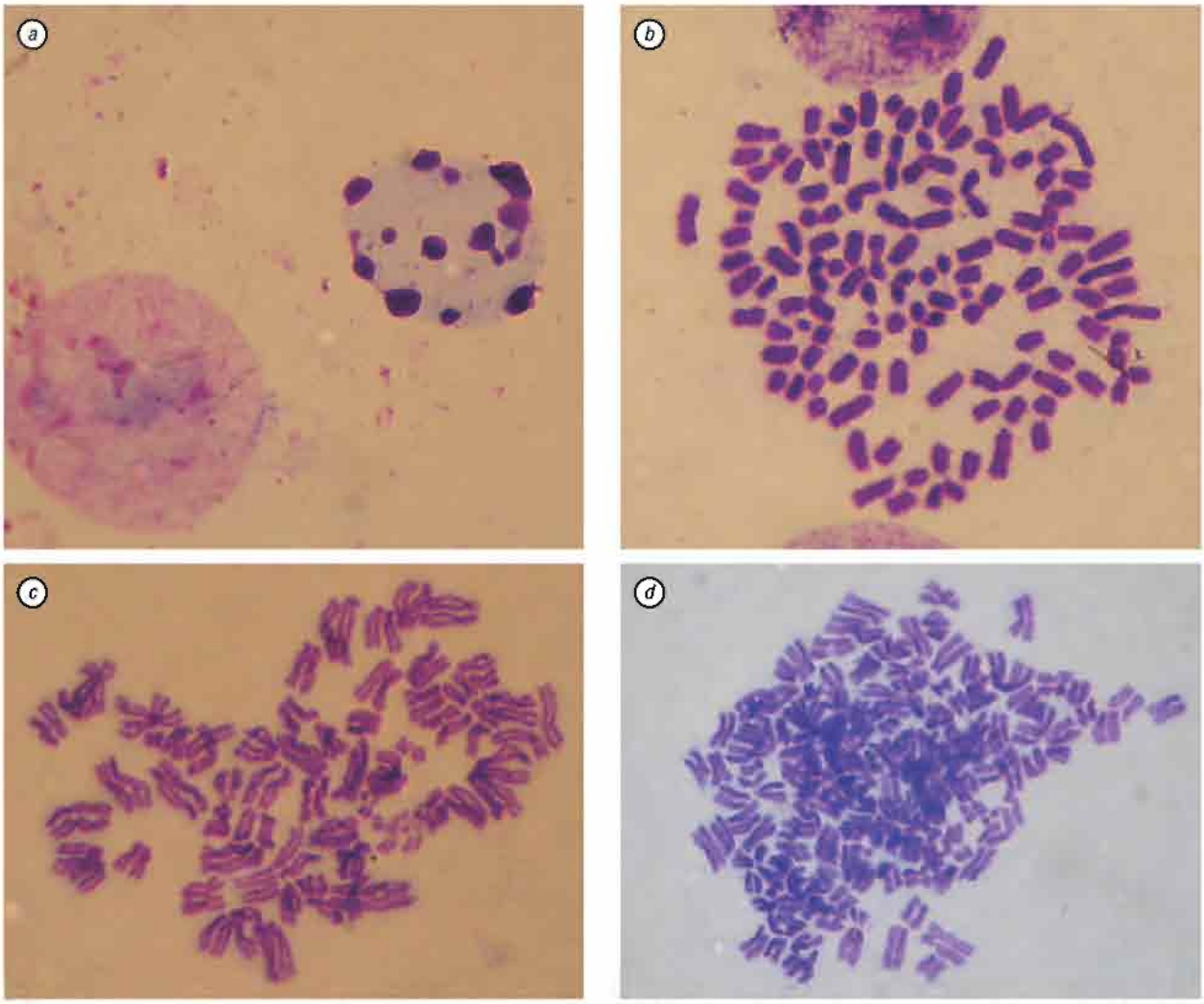

Fig. 3. Apoptosis and pathologies of mitosis in LL/BV/IFN cells: *a* — apoptotic cell; *b* — polyploidy; *c*, *d* — endoreduplication with different numbers of chromosomes (magnification × 100)

Treatment of LL cells with exogenous IFN-β resulted in a statistically significant reduction of cytoplasmic expression of $p19^{ARF}$ protein — 250 ± 27 points in control *vs* 110 ± 15 points in LL + IFN-β cells (see Table 4, Fig. 4).

Immunocytochemical analysis of $p21^{WAF1}$ protein expression in LL cells after their transduction with BV or BV/IFN showed that infection of LL cells with any type of baculovirus vector leads to an increase of nuclear expression of this protein.

Transfection of LL cells with BV, as well as the treatment of these cells with exogenous IFN-β, led to increased number of $p21^{WAF1}$-positive cells by 40% and the level of expression this protein — 30 ± 5 points in control *vs* 100 ± 10 in LL/BV cells and *vs* 120 ± 12 in LL + IFN cells ($p < 0.01$) (see Table 4).

Transduction of LL cells with BV/IFN enhanced the effects of BV and caused a significant increase of p21 protein expression level (30 ± 5 points in the control cells *vs* 270 ± 20 points in LL/BV/IFN) and the number of $p21^{WAF1}$-positive cells up to 97 ± 2% (see Table 4).

Immunocytochemical analysis of the expression of transcription factors (Twist, Slug) and molecules of adhesion (E-cadherin and N-cadherin) in LL cells transduced with BV or BV/IFN showed that transduction of tumor cells with BV/IFN rapidly (in 72 h) led to the disappearance of nuclear and cytoplasmic expression of Slug (Fig. 5) and significant increase of cy-

Table 4. The expression of cell cycle regulation proteins, EMT transcription factors and cell adhesion molecules in LL cells transduced with BV/IFN

Antigen	LL control cells		LL/BV cells		LL/BV/IFN cells		LL + IFN-β cells	
	Subcellular localization of antigen							
	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
$p19^{ARF}$	140 ± 20	250 ± 27	130 ± 22	270 ± 30	270 ± 20*	50 ± 5*	105 ± 10	110 ± 15*
$p21^{WAF1}$	30 ± 5	–	100 ± 10*	–	270 ± 20***	–	120 ± 12*	–
Twist	180 ± 20	240 ± 25	130 ± 15	250 ± 30	0**	200 ± 25	150 ± 15	220 ± 25
Slug	280 ± 20	270 ± 28	260 ± 30	280 ± 25	0***	0***	270 ± 30	250 ± 20
E-cadherin	–	100 ± 15	–	110 ± 10	–	270 ± 15**	–	120 ± 10
N-cadherin	–	120 ± 10	–	100 ± 15	–	110 ± 12	–	115 ± 12

Note: the level of expression of antigens is presented in points on H-score system. Difference *vs* control LL cells is statistically significant; *$p < 0.01$, **$p < 0.005$, ***$p < 0.001$.

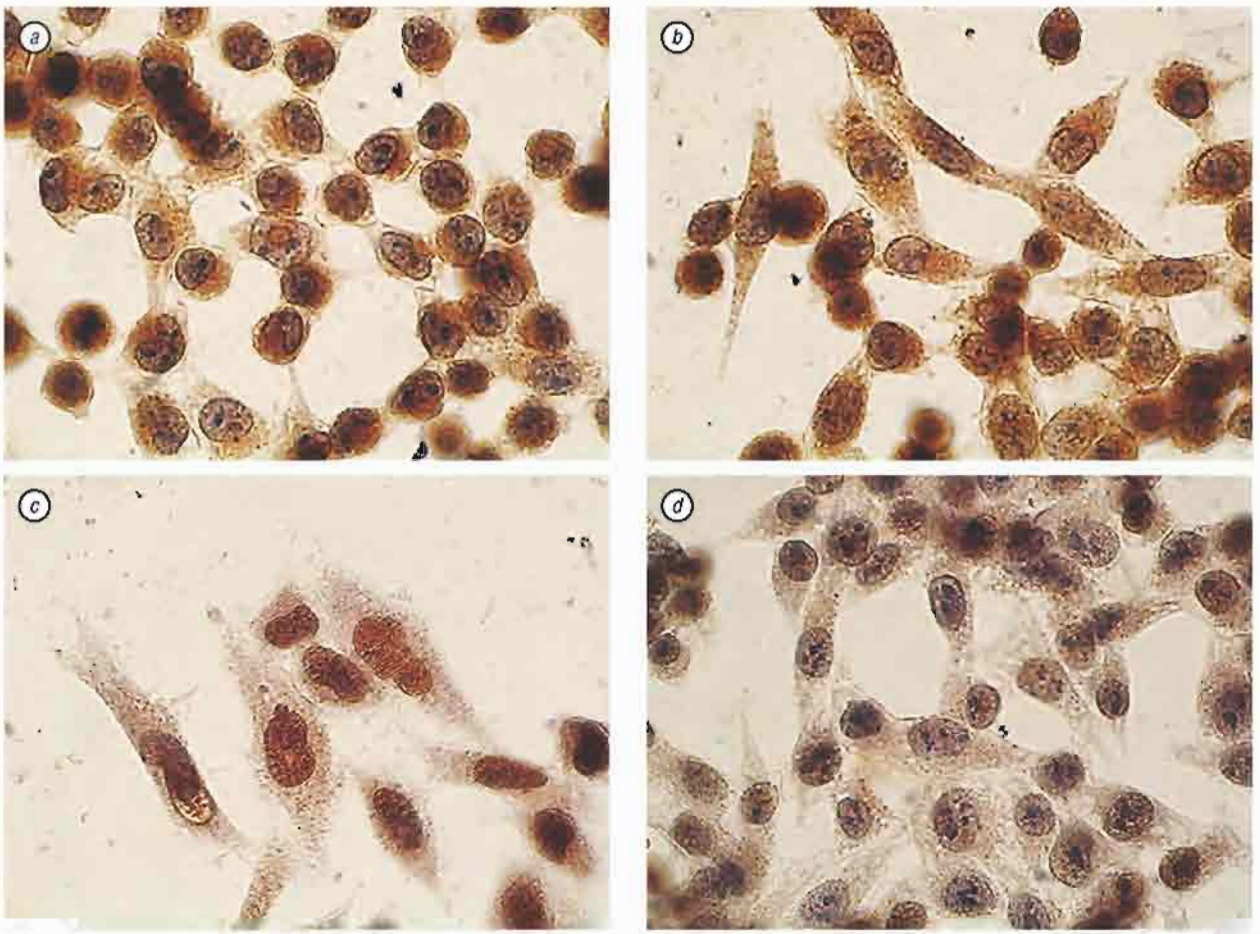

Fig. 4. Expression of $p19^{ARF}$ protein in LL cells transduced with *ifn-β* gene in BV (magnification × 100): *a* — control LL cells; *b* — LL/BV; *c* — LL/BV/IFN; *d* — LL + exogenous IFN-β

toplasmic expression of E-cadherin (Fig. 6) — from 100 ± 15 points in control LL cells to 270 ± 15 points in LL/BV/IFN cells (see Table 4). At the same time, cultivation of LL cells in the presence of exogenous IFN-β for 72 h had no significant effect on the expression of these proteins (see Table 4).

DISCUSSION

Many studies give evidence of antitumor efficacy of gene therapy with cytokines, in particular *ifn-β* cloned in viral vectors [5, 7, 10–12, 14]. Interest in baculoviruses as potential vectors for recombinant molecules is motivated by their ability to provide genetic transduction elements of significant size in mammalian cells and to control the expression and synthesis of large quantities of recombinant proteins [23]. A significant advantage of the recombinant baculovirus system is the lack of replication of the virus and a low level of synthesis of viral proteins in mammalian cells, as well as low cytopathogenic effect of baculovirus in these cells [24].

We have recently shown that LL cells transduced with *ifn-β* gene in baculovirus vector possesses lower tumorigenicity and metastatic potential *in vivo*, but the mechanisms of these effects were not thoroughly studied. Therefore, we examined the spectrum of characteristics of these cells *in vitro*, associated with malignancy. Given that the increase in malignancy is closely related to cell ploidy [25] and activation of EMT process [26] we have studied cytogenetic characteristics of LL cells and their immunophenotype associated with EMT. For comparison of possible mechanisms of action of BV/IFN, exogenous and endogenous (recombinant) IFN-β were used.

The rate and density of the cell culture growth are important characteristics of tumor cells. Tumor cells are also characterized by a high degree of autonomy and the ability to produce their own growth factors and nutrients necessary for their survival. The ability of cells to grow in serum-free medium is one of the indicators of cell malignancy [27]. Another important indicator is their ability to migrate.

The results of our studies have shown that transduction of LL cells with BV/IFN results in a significant change of their biological properties: reduction of cell growth rate and density, inhibition of cell growth in a serum-free medium and suppression of migration activity. If one takes into account the suppression of LL cells colony formation in agar after *ifn-β* gene transtuction [7], these data suggest the suppression of malignancy of BV/IFN transduced LL cells.

The study of the changes of cytogenetic characteristics of LL cells after their transduction with BV/IFN or after the treatment with exogenous IFN-β showed that the transduction of LL cells with BV/IFN was accompanied by an increase the number of cells with micronuclei and frequency of ACCC and pathology of mitosis. At the same time, culturing of tumor cells

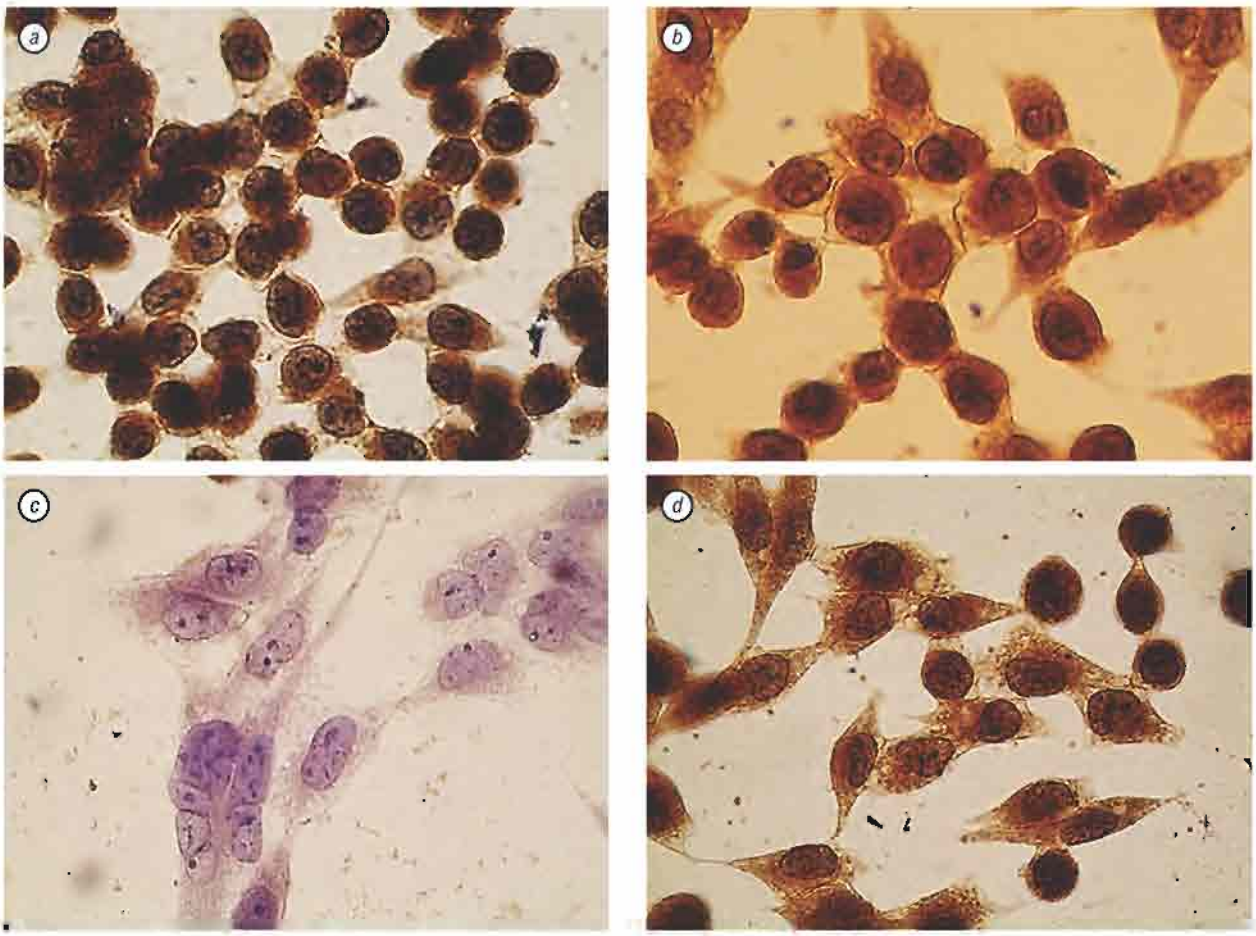

Fig. 5. Suppression of expression the transcription factor Slug in LL cells after their transduction with BV/IFN or treatment of exogenous IFN-β (magnification × 100): *a* — control LL cells; *b* — LL/BV; *c* — LL/BV/IFN; *d* — LL + exogenous IFN-β

with IFN-β leads to increase the frequency of nuclear Protr and CA. All these data suggest on significant genotoxic effect of exogenous IFN-β. Transduction of LL cells with BV/IFN was also accompanied with a significant increase of apoptosis rate and by suppression of proliferation. There results are in accordance with the published data concerning antiproliferative and genotoxic effects of type I IFNs, including IFN-β [28, 29].

The studies of the expression of some proteins associated with cell cycle regulation, invasive and migration potential *in vitro* of lung cancer cells can explain some mechanisms that ensure the changes of biological properties of LL cells after their transduction with BV/IFN and the differences between effects of baculovirus vector with *ifn-β* gene and exogenous IFN-β.

$p19^{ARF}$ protein accumulates mainly in the cell nucleus where it forms a stable complex with Mdm2. This interaction provides oncosuppressive effect of $p19^{ARF}$ via activation of apoptosis [30]. In the cytoplasm $p19^{ARF}$ interacts with the protein Pex19p. This complex is transported into the nucleus that leads to inactivation of wild type p53 [31]. Significant decrease of $p19^{ARF}$ cytoplasmic level along with an increase of nuclear expression of this protein in LL/BV/IFN cells could lead to the rise of apoptosis rate and to normalization of cell cycle regulation in these cells. Indeed, the results of cytogenetic analysis of LL cells showed the increase of apoptosis rate in tumor cells after transduction with BV/IFN. At the same time, treatment of LL cells with exogenous IFN-β leads to a significant inhibition only of the cytoplasmic expression of $p19^{ARF}$ protein.

$p21^{WAF1}$ is considered as oncosuppressor and plays an important role in activation of apoptosis. In addition, this protein can be expressed in cell nuclei in response to genotoxic stress [32]. In our opinion, increase the level of expression of $p21^{WAF1}$ in LL/BV cells is associated with genotoxic effect, which is caused by the penetration of baculovirus vector into tumor cells. Transduction of LL cells with BV/IFN strengthened genotoxic effect by activation of cytotoxic and antiproliferative effects of IFN-β.

Cell adhesion molecules E-cadherin, N-cadherin and transcription factor Slug and Twist play an important role in the process of EMT [33].

It was shown that the increase of E-cadherin expression is inversely correlated with progression of tumor growth and metastasis independently of tumor histogenesis [34]. Increased N-cadherin expression in tumor cells is associated with an increase in their invasive potential [35]. Transcription factor of EMT Slug functions as a direct repressor of E-cadherin *in vitro* and *in vivo* and is involved in the process of ensuring the resistance of cells to apoptosis [36]. Moreover, increased expression of transcription factor Twist induces an increased mobility of tumor cells and provides antiapoptotic effect [37].

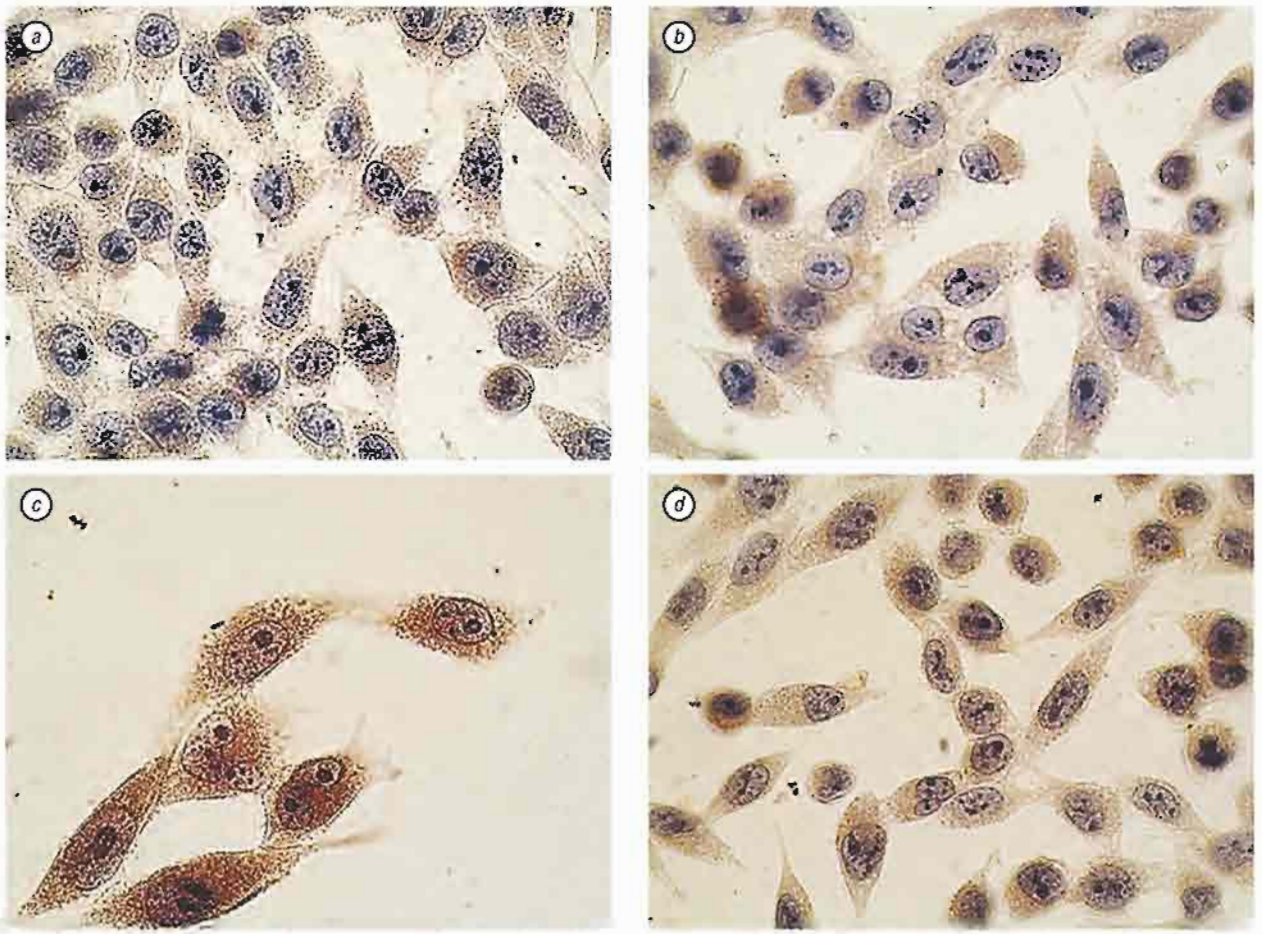

Fig. 6. Increase of cytoplasmic expression of E-cadherin in LL cells transduced with BV/IFN but not in cells treated with exogenous IFN-β (magnification × 100): *a* — control LL cells; *b* — LL/BV; *c* — LL/BV/IFN; *d* — LL + exogenous IFN-β

The changes in the expression of E-cadherin, N-cadherin and transcription factor Slug and Twist in LL/BV/IFN cell indicate a significant inhibition of EMT and reversion of carcinoma cells to normal epithelial phenotype.

Considering all obtained results it can be noted that the transduction of lung carcinoma cells with BV/IFN more efficiently suppresses malignancy of tumor cells than the treatment of these cells with exogenous IFN-β. Such effects may be caused by the presence of cells that permanently produce recombinant biologically active IFN-β in a population of LL cells transduced with BV/IFN [7].

It should be noted that all the observed effects occur within 72 h after *ifn*-gene transduction of LL cells. As it was shown earlier [7], transduction of lung carcinoma cells with *ifn-β* gene in recombinant baculovirus results in the production of IFN-β as secretory protein, and it may also be accumulated in specialized intracellular vesicles. Currently, the effect of IFN-β on tumor cells through cell surface receptors and IFN signaling molecules is well-known, but effects of IFN-β stored in such vesicles that can deliver interferon to cytosol of tumor cells by mechanism of endocytosis, have not been extensively investigated. It'll be of interest to determine biological effects of endogenously produced IFN-β stored in intracellular vesicles of BV/IFN-transduced tumor cells.

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