# Loss of Malignancy in Mouse Melanoma Cells by Long-Term Impact of Interferon-Beta *In Vitro* is Associated with N- and VE-Cadherins Suppression without Inhibition of Expression of EMT-Associated Twist and Slug Proteins

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**Abstract:** *Aim*: To study the effect of long-term impact of mouse interferon-beta (IFN-b) on the behavior of mouse melanoma cells *in vitro* and *in vivo* and the expression of epithelial-mesenchymal transition (EMT) associated proteins.

*Materials and Methods*: Studies were performed on mouse B16 melanoma cells as a tumor model (MM-4 cell line). Immunocytochemical and tumor cell biology approaches have been used in this study.

*Results*: Long-time treatment of MM-4 melanoma cells with low-dose IFN-b (1/2 of IC50) leads to change their morphology, significant inhibition of cell growth and plating efficiency, suppression of cell migration and anchorage-independent growth in semisolid agar. Moreover, IFN-modification of melanoma cells is accompanied by the significant suppression of their malignancy *in vivo*: growth of tumor induced by IFN-treated cells has inhibited on 50% and growth of metastases - on 90%. Also, IFN-modification of MM-4 cells affects on the expression of proteins involved in cell cycle regulation and inhibits expression of some molecules of adhesion (N-, VE-cadherins), but not influence on the expression of EMT-associated Twist and Slug proteins and E-cadherin.

*Conclusions*: Long-term impact of mouse IFN-beta in low dose on melanoma cells *in vitro* changes their phenotype and inhibits their proliferative potential, signs of malignancy *in vitro,* tumorigenicity and metastatic ability *in vivo*. Loss of malignancy is associated with inhibition of N- and VE-cadherins expression, but not associated with the change of expression and subcellular localization of E-cadherin and EMT transcription factors Twist and Slug.

**Keywords:** Cadherins, anchorage-independent growth, changes of phenotype, tumorigenicity, metastasis.

## 1. INTRODUCTION

Malignant melanoma is an aggressive form of skin cancer and frequently can travel to the lymph nodes, skin, lung, liver and brain. The surgery remains the basic treatment for melanoma patients [1]. While the susceptibility of melanomas to conventional chemo- and radiotherapy is rather low, interferons (IFN), primarily alpha/beta subtypes, are considered as fairly effective means to combat recurrent melanoma [2,3]. The IFNs are cytokines with pleiotropic properties that may be advantageous as the agents of adjuvant therapy in the treatment of various solid tumors and leukemia. It is accepted that IFN-b facilitates tumor regression in several ways: by regulation of cell proliferation, differentiation, antigen expression, apoptosis [4] and by activating few components of innate and adaptive immunity: natural killer cells (NK) and macrophages [5], dendritic and T-cells. Moreover, IFNs are potent stimulators of adaptive immune responses and inhibit tumor angiogenesis [6]. Several studies revealed significant differences in the antiproliferative effects of the different IFN types. In some experiments *in vitro*, the antiproliferative effects of IFN-b on melanoma cells were superior to that of other IFN types [7].

Earlier it has been shown that IFN suppresses malignancy of murine cells transformed with viral oncogenes and changes the methylation patterns of viral and/or cellular genes. Long-term *in vitro* culture of human tumor cells in the presence of IFN-alpha might contribute to the reversion of the malignant phenotype [8].

While IFN-alpha is prevalent in the therapy of melanoma, several studies demonstrates that IFN-beta may be more effective although the underlying mechanisms of the different efficacy of various IFN types have not been yet elucidated [9,10]. Earlier, we have shown [11] that transduction of mouse melanoma cells with IFN-beta gene resulted in decrease of their proliferative and tumorigenic potential *in vitro* and *in vivo*.

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It is well known that IFN can alter a variety of properties of tumor cells relevant to their malignant behavior [11-14]. However, the principal mechanisms contributing to the loss of malignancy caused by IFN-b remain unknown. Previously, we have found that the reduced malignancy of tumor cells treated with IFN for a long time may be associated in changed patterns of the expression of proteins associated with epithelial-mesenchymal transition [15]. We believe that *in vitro* models of long-term treatment of tumor cells with IFN are relevant to the clinical schedules of long-term courses of IFN therapy that may be more advantageous in reinforcing the advantageous effects of IFN.

The aim of the present study was to analyze the effects of long-term treatment of mouse melanoma cells *in vitro* with IFN-b on biological characteristics of melanoma cells, their phenotype and tumorigenicity *in vivo*.

## 2. MATERIALS AND METHODS

### 2.1. Cell Lines

Cells of mouse melanoma (MM-4 cell line) [16] were obtained from the Bank of Cell Lines of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (IEPOR NASU). Cells of MM-4/IFN subline were obtained in our laboratory by long-term culture of MM-4 cells with low-dose (500 IU/ml) of recombinant IFN-beta (Sigma, USA, Cat.N 19032) for 185 days. MM-4 and MM-4/IFN cell lines were maintained in plastic flasks (TPP, Italy) in DMEM supplemented with 4 mmol/l L-glutamine (PAA, Austria), 10% newborn calf serum (NCS) (PAA, Austria), and 40 μg/ml gentamicin (Sigma, USA). The cells were incubated at $37^0$C in a humidified atmosphere with 5% $CO_2$. Cells were detached with EDTA solution (BioTestMed, Ukraine) by the standard method. Cell density was counted in hemocytometer and their viability was determined in trypan blue (Bio-Rad, USA) exclusion assay.

### 2.2. Study of Cell Morphology

The flasks with MM-4 cells were viewed in inverted microscope Axiovert 25 (Carl Zeiss, Germany) equipped with digital camera (Canon PowerShot A640, UK) at 100-320-fold magnification. The specimens of live cells were photographed, and the images were analyzed using Shortcut to Remote Capture software.

### 2.3. Analysis of Growth Kinetic of Tumor Cells

All experiments were started from stock cultures maintained in exponential phase of growth. Cell suspensions ($1x10^4$ cells/ml) were seeded into 96-well culture plates, 100 μl per well and incubated for 24 h at $37^0$C. Then the medium was replaced with fresh medium containing different concentrations of recombinant IFN-beta (6200 IU/ml, 3100 IU/ml, 1550 IU/ml, 775 IU/ml, 388 IU/ml, 194 IU/ml, 97 IU/ml). Plates were incubated for 72 hours at $37^0$C and 5% $CO_2$. Then the IFN-containing medium was removed and cell count was determined by colorimetric assay with crystal violet [17]. All experiments were performed in triplicate and the results were expressed as inhibitory rate (IR):

$$IR = (1 - A_{540}(\text{experiment})/A_{540}(\text{control})) \times 100\%$$

In another experiments cells ($1x10^4$/ml) were seeded in 24-wells plate in 1 ml of DMEM medium (PAA, Austria) supplemented with 10% NCS (PAA, Austria) and 40 μg/ml gentamicin (Sigma, USA). The cells were incubated at $37^0$C in a humidified atmosphere with 5% $CO_2$ for 8 days. Every day cells were detached with EDTA solution (BioTestMed, Ukraine) from three wells at each time point, stained with trypan blue solution and counted in hemocytometer. All the experiments were repeated twice.

Cell population doubling time (Td) was determined by the formula:

$$Td = \frac{T}{(\text{Log Nf} - \text{Log N0}) \times 3.3219}$$

wherein

T = time of cell exponential growth (h)

3.3219 = coefficient of transforming log to ln

### 2.4. Scratch Assay *In Vitro*

The cells ($1x10^5$/ml) were seeded in 35 mm plastic dishes (TPP, Italy) in DMEM medium (PAA, Austria) supplemented with 10% NCS (PAA, Austria), 40 μg/ml gentamicin (Sigma, USA) and incubated at $37^0$C in a humidified atmosphere with 5% $CO_2$ until cells reach 100% confluence to form a monolayer. We used a p200 pipet tip to create a scratch off the cell monolayer. In each dish the medium was changed to remove debris and to smooth the edge of the scratch. After

that, the migration of cells in the ''scratch'' was photographed with phase contrast microscope. The first image was made at 0 hours and the following images were taken at intervals of 3 h. The images were analyzed and the time of incubation of cells required to close the scratch was determined [18].

### 2.5. Platting Efficiency Assay of Cells *In Vitro*

To determine the effectiveness of the platting on the substrate, MM-4 cells were seeded in paired wells of 6-well plates (TPP, Italy) at a density of 200 cells per well in complete growth medium (DMEM with 10% NCS and 40 μg/ml gentamicin) and incubated at $37^0$C in 5% $CO_2$. After 14 days, colonies of the cells were stained with the crystal violet dye (Sigma, USA) [17]. Number of colonies and their size were determined in each well using binocular magnifier BM-51-2 (LOMO, Russia). The size of the colony was defined as small (10-20 cells), medium (21-50 cells) and large (more than 50 cells).

### 2.6. Semisolid Agar Colony Assay

Base layers of 0.5% agar (Difco, USA) prepared in DMEM medium plus 10% NCS and 40 μg/ml gentamicin were added in parallel wells 6-well plates, 4 mL each. A 2 ml layer of 0.33% agar containing MM-4 cells ($2x10^3$) in DMEM medium with 10% NCS and 40 μg/ml gentamicin was overlaid. The plates were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. The number and the size of colonies were counted in each well in 14 days after plating using binocular magnifiers BM-51-2 (LOMO, Russia). In some experiments, the colonies were stained with 0.2% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide) solution (Sigma, USA) or 0,4% solution of neutral red (Sigma, USA) for 3 h [19, 20].

### 2.7. Immunocytochemical Assay

The proliferative activity and phenotype of MM-4 melanoma cells modified by long-term culture in the presence of recombinant IFN-beta were determined by immunocytochemical evaluation of cells expressing PCNA (marker of DNA replication), protein involved in cell cycle regulation – p21 (NeoMarkers, USA), transcriptional regulators – Twist and Slug (GenTex, USA), and E-cadherin, VE-cadherin, (Thermo scientific, USA), N-cadherin (BioLegend, USA) - molecules of adhesion. Cells were grown on the coverslips for 48 hours and analyzed by the standard method [21] using monoclonal antibodies against relevant antigens. For visualization, Ultra Vision LPValue Detection system was used. DAB Quanto (3,3-diaminobenzydyne) (Thermo Scientific, USA) was used as a chromogen.

Immunocytochemical reactions were assessed semiquantitavely using H-score accounting for the proportion 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 staining cells) to 300 (diffuse intense staining of cells), and H score was calculated as (% of cells stained at intensity 1×1) + (% of cells stained at intensity 2×2) + (% of cells stained at intensity 3×3). H-score between 0 and 300 was obtained where 300 corresponded to 100% of intense staining [22].

### 2.8. Study of Tumorigenicity and Metastasis of Melanoma Cells *In Vivo*

Tumorigenic and metastatic properties of MM-4 and MM-4/IFN cells were determined in experiments *in vivo* in C57BL/6 mice aged 7-8 weeks with body mass of 20-22 g in animal facility of R.E. Kavetsky IEPOR NAS of Ukraine. The mice were kept under standard conditions with granulated feed and water ad libitum. The animals were euthanized using either anesthesia. MM-4 control cells and MM-4 cells treated 500 IU/ml IFN-beta for 190 days (MM-4/IFN) were used for experiments *in vivo*.

The suspension of control and modified MM-4/IFN cells (at a dose of $3x10^5$ cells per mouse in physiologic saline) were injected to animals intramuscularly (i/m) in a volume of 0,1 ml or intravenously (i/v) in a volume of 0,2 ml (experimental metastasis).

Ten mice in each group were used in experiments *in vivo*. The experiment was repeated twice. The dynamics of tumor growth in mice was studied by measuring the tumor diameter every 3 days. Numbers of metastases were calculated on 28 days after transplantation of tumor cells. Volume of tumors and metastases in $mm^3$ was determined by the formula $V = D^3 \times 0.52$, where V - volume of metastases, D - diameter of a single tumor or metastases.

### 2.9. Statistical Analysis

Statistical data processing was performed by the Student t-test or nonparametric U-test of Wilcoxon-Mann-Whitney using STATISTICA 6.0.

## 3. RESULTS AND DISCUSSION

Cancer cells are characterized by highly autonomous growth *in vitro* that typically correlates with their malignancy *in vivo* [21]. We have shown that the properties of melanoma cells, exposed for a long time to recombinant IFN-b, have undergone major changes: the cells become more pronounced epithelioid morphology, decrease in the rate and density of cells growth and suppression of migration of melanoma cells. Their plating efficiency and colony formation in semi-solid agar were sharply reduced. Moreover, treated of melanoma cells by IFN-b leads to significant inhibition of expression molecules of adhesion and proteins involved in the process of angiogenesis.

### 3.1. Sensitivity of Mouse Melanoma MM-4 Cells to Recombinant IFN-Beta

Antitumor effect of recombinant mouse IFN-beta was evaluated in antiproliferative assay.

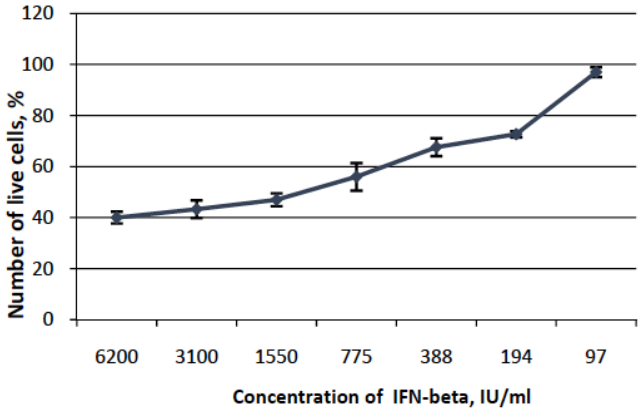


**Figure 1:** Antiproliferative activity of recombinant IFN-beta in MM-4 cells.

The assay revealed antiproliferative effect of IFN-beta on MM-4 cells with IC50 of 1100 ±80 IU/ml (Figure **1**). The sensitivity of MM-4 cells to recombinant IFN-beta in our study coincides with sensitivity murine L929 cells to natural alpha/beta-IFN [22]. In further experiments, IFN-beta was used at a dose less than IC50 (500 IU/ml).

### 3.2. Morphology of MM-4 and MM-4/IFN-Beta Cells

Morphological studies have shown that the long-term incubation of melanoma cells with IFN-beta changes their morphology: cells acquired more epithelioid morphology with increasing their size and growth area (Figure **2**). These changes have accumulated to 165 days of cultivation in the presence of IFN-b.

### 3.3. The Growth Kinetics of MM-4 and MM-4/IFN-Beta Cells

The kinetics and density of cell growth, doubling time of cell population are considered as very important features of tumor cells. Therefore, we attempted to study the kinetics of MM-4 cell growth as well as their morphology in comparison with their counterpart cultured with IFN-b at low concentrations up to 165 days. We have noticed the gradual accumulation of the changes in morphology and cell growth. The growth kinetics of MM-4 and MM-4/IFN-beta cells analyzed within 8 days is presented in Figure **3**. The MM-4/IFN-beta cells demonstrated slower exponential growth as compared with original MM-4 cells (7 days vs. 5 days) and lower cell count and cell density at the end of the exponential phase (130.5 ± 5.2 × $10^5$ cells per well and 1.6x$10^5$ cells/$cm^2$ vs. 195,8 ± 25,2× $10^5$ cells per well

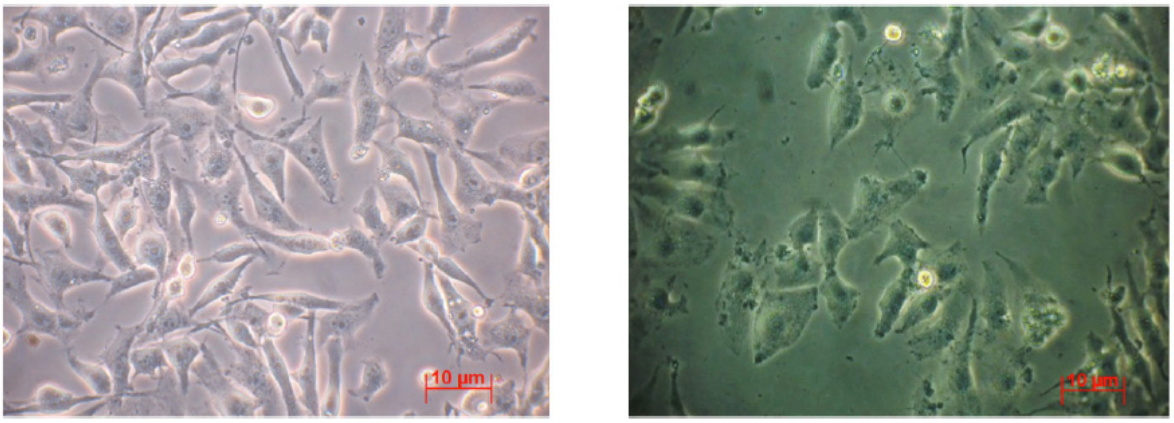


**Figure 2:** Morphology of MM-4 cells treated with IFN-b. **A**. Untreated MM-4 cells; **B**. MM-4 cells treated with 500 IU/ml of IFN-b for 185 days (magnification x320).

and 2,5x10$^5$ cells/cm$^2$). The population doubling time of control MM-4 cells was estimated as 22 h compared with 36 h in MM-4/IFN-beta cells.

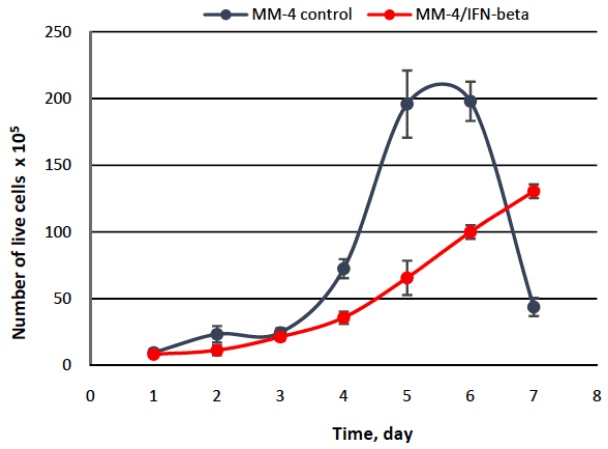


**Figure 3:** The growth kinetics of MM-4 and MM-4/IFN-beta cells.

It is known that intracellular mechanisms that ensure suppression of cell proliferation and activation of contact inhibition in normal cells involves differentiation or concentration-dependent inhibition of proliferation rate [25]. The malignant cells demonstrate dysregulation of proliferation, differentiation and cell motility [26]. Furthermore, increase in the density of the cell population lead to contact-activated migration of cancer cells [25]. In malignant cells, defects in receptor molecules providing intercellular contacts might be one of the factors contributing to the failure of the contact-dependent inhibition of cell growth. The impairment of synthesis or post-translational modification of these proteins or activation of certain oncogenes leads to the suppression of normal regulation of proliferation and differentiation in transformed cells [27].

### 3.4. Plating Efficiency and Anchorage Independent Growth of MM-4 and MM-4/IFN-Beta Cells

Significant decrease in the rate and density of MM-4/IFN-beta cells growth reflects not only the inhibition of cell growth by IFN-b, but indicates a change in their biological characteristics possibly suggesting the inhibition of their malignant potential. To ensure the changes of biological properties, we next compare the plating efficiency and the anchorage independent growth of MM-4 and MM-4/IFN-beta cells since effectiveness of colony formation of cancer cells on plastic substrate and colony formation in agar are the important indicators of the ability of cells to autonomous growth.

The plating efficiency was assessed as the percentage of cells that proliferate as surface-attached colonies when plated at low density (Figure **4**). It has been shown that IFN-b treatment of MM-4 cells for 185 days resulted in suppression of their plating efficiency of these cells decreasing colony-forming activity from 77.3% ± 3.0% to 32.0 %± 1.8 %.

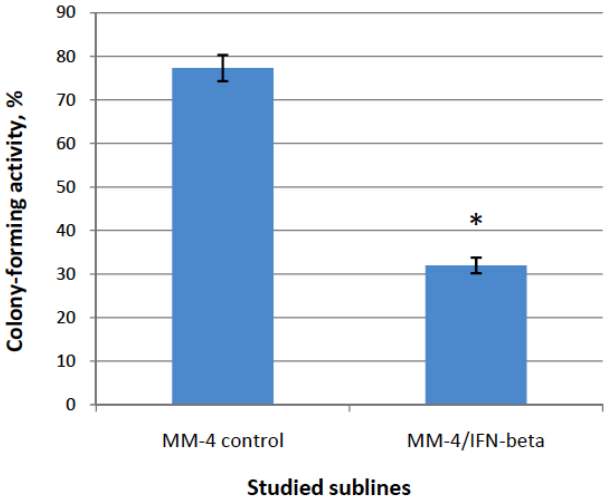


**Figure 4:** Inhibition effect of the mouse IFN-beta on plating efficiency of MM-4 cells *in vitro*. *p < 0.001 compared to control.

In normal cells, there is a plurality of external and intracellular signals that ordinarily regulate their growth. In contrast, the malignant cells through a series of genetic and epigenetic changes gain the ability to anchorage-independent growth, which is one of the hallmarks of cell transformation. The colony-forming *in vitro* assay is considered as the most precise method for detecting malignant transformation of cells [19]. The ability of tumor cells to form colonies in semisolid agar has been correlated with tumorigenicity and metastatic potential [20].

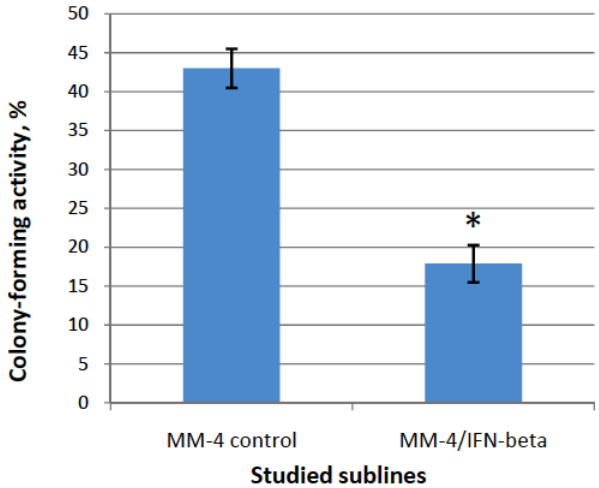


**Figure 5:** Colony-forming activity of MM-4 control and cells treated with IFN-beta for a long time in semisolid agar. *p< 0.001 compared to control.

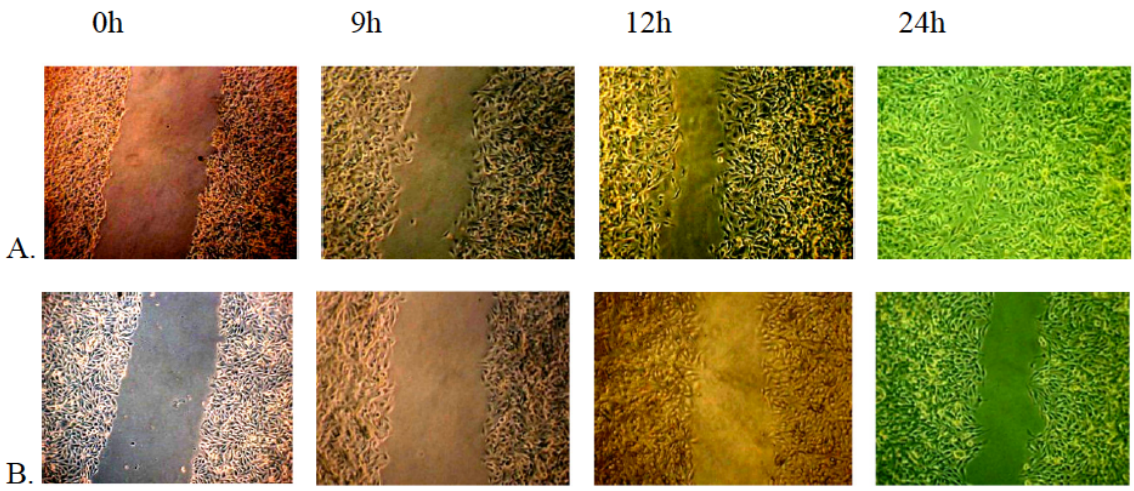


**Figure 6:** Analysis of melanoma cells migration by in *vitro* in scratch assay. **A**. MM-4 control, **B**. MM-4/IFN.

Our study of the colony forming activity (CFA) in semisolid agar of melanoma cells have shown that long-term treatment of these cells with low dose of IFN-b results in more than two-fold decrease in the number of colonies indicating the decreased tumorigenicity of the cells.

### 3.5. Analysis of Melanoma Cells Migration *In Vitro*

One of the simplest methods of analysis of tumor cell migration is *in vitro* scratch assay. This method has several advantages. Primarily, cell migration *in vitro* to some extent is similar to their migration *in vivo*. Moreover, it can be used to study the mechanisms of regulation of cell migration through cell interaction with extracellular matrix and with each other [28].

Figure **6** visualized the results of *in vitro* scratch assay to track cell migration in the leading edge of the scratch. The analysis of the images showed that the MM-4 cells that were long-term treated with IFN-beta (B) migrated at a slower rate compared with the untreated control cells (A) in the leading edge of the scratch under the same conditions.

It was shown, that MM-4 control cells close the scratch in the 24 hours after the start of the experiment. At the same time, MM-4/IFN-beta cells restored the monolayer only after 60 hours. Thus, comparison of the untreated cells and those treated with the recombinant IFN-beta also showed significant inhibition of cell migration by this cytokine.

### 3.6. Phenotype Changes of MM-4/IFN-β Cells

Next, we have analyzed by immunocytochemical assay the expression of several proteins involved in DNA replication in eukaryotic cells. Proliferating cell nuclear antigen (PCNA) representing an accessory protein of DNA polymerase delta might be involved in DNA repair, chromatin assembly and DNA methylation and is considered as a useful marker of DNA synthesis.

**Table 1: Immunocytochemical Analysis the Expression of Some Cellular Proteins**

| Marker | MM-4 control cells | | MM-4/IFN-beta cells | |
|---|---|---|---|---|
| **Localization** | **Nucleus** | **Cytoplasm** | **Nucleus** | **Cytoplasm** |
| PCNA | 120 ± 15(60%) | 0 | 15 ± 2(15%)* | 0 |
| $p21^{Waf1}$ | 0 | 0 | 100±8** | 0 |
| Twist | 50 ± 8 | 200 ± 28 | 130 ± 11* | 200 ± 32 |
| Slug | 100 ± 17 | 200± 10 | 130 ± 21 | 200± 30 |
| N-cad | 0 | 150 ± 12 | 0 | 50 ± 5** |
| E-cad | 0 | 180 ± 20 | 0 | 170 ± 16 |
| VE-cad | 0 | 200 ± 25 | 0 | 0** |

The level of expression of antigens presented in points on a H-score system.
*p < 0,01, **p < 0,005 compared to control.

It is of interest that PCNA may also interact with proteins involved in cell cycle regulation such as cyclin-dependent kinase inhibitor p21 (Cip1/Waf1), which binds to PCNA blocking its activity required for DNA replication. Moreover, p21 affects the interaction with other PCNA-binding proteins [29]. Immunocyto-chemical study with monoclonal antibodies against PCNA and p21(Waf1) demonstrated that staining of MM-4/IFN-beta cells with anti-PCNA antibody drastically decreased while $p21^{Waf1}$ positivity became evident in MM-4/IFN-beta cells in contrast to $p21^{Waf1}$ negative original MM-4 cell line (Table **1**).

In recent years, considerable attention is paid to the role of EMT in the control of malignant growth and metastasis of solid tumors, including melanoma [30, 31, 32]. Cadherins are a major class of molecules of cell adhesion, which form the transmembrane part of the adherent junctions. E- (epithelial), N- (neural) and VE- (vascular endothelial) cadherins are the best studied representatives of this class [33]. Several studies have revealed that only invasive dermal and uveal melanoma cells express VE-cadherin as opposed to low aggressive cancer cells. VE-cadherin expression is also related to neoangiogenesis triggered by aggressive melanomas [34]. Since cadherins represent important EMT markers, regulation of their expression may be related to the malignant potential of cells under study. That is why we focused on the changes in expression of EMT-associated proteins after long-term culture in presence of IFN-beta *in vitro*.

Our results showed that the VE-cadherin in the untreated MM-4 cells was detected in 100% of cells. Long-term treatment of melanoma cells by IFN-beta leads to loss of its expression in MM-4/IFN-beta cells (Figure **7**).

In the growth of melanoma, tumor cells often lose the expression of E-cadherin. At the same time the N-

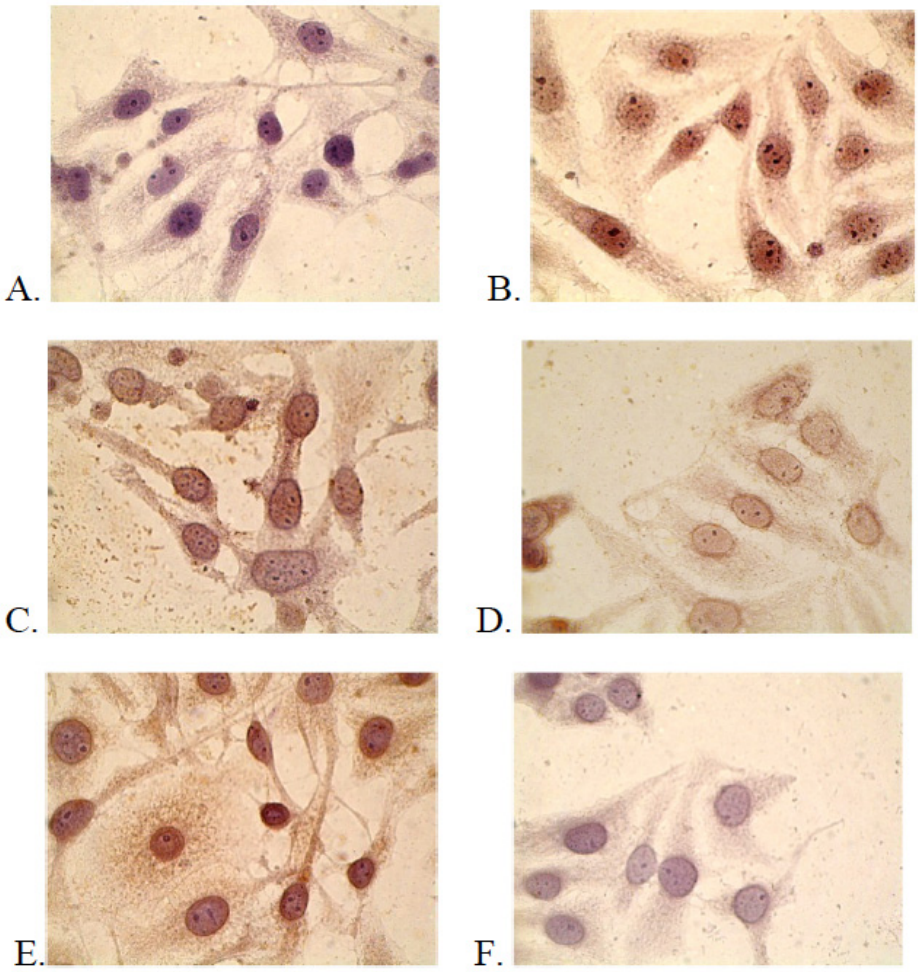


**Figure 7:** Changes in the expression of some cellular proteins.

The expression of p21(Waf1) protein in MM-4 control cells (**A**) and MM-4/IFN-beta cells (**B**); N-cadherin in MM-4 control cells (**C**) and MM-4/IFN-beta cells (**D**); VE-cadherin in MM-4 control cells (**E**) and MM-4/IFN-beta cells (**F**). (magnification x1000).

cadherin level of expression *in vitro* and *in vivo* is increased resulting in enhancement of tumor-stroma cell adhesion, tumor cell migration and invasion. The role of N-cadherin in melanoma metastasis is also suggested [34]. We have shown that the long-term exposure of MM-4 melanoma cells to IFN-beta leads to significant decrease both in level of expression and the number of cells expressing N-cadherin approximately by 50% (Table **1**).

It is known that the loss of E-cadherin expression in carcinoma cells is associated with their invasiveness and dedifferentiation [35]. Nevertheless, our results demonstrated similar expression of E-cadherin both in MM-4 control cells and modified MM-4/IFN-beta cells practically in 100 % of cells.

Transcription factors Slug (zinc finger protein) and Twist in mouse melanoma B16 cells were identified in the cytoplasm as well as in the nucleus. Slug expression patterns suggest its role in tumor progression [36]. This factor is supposed to act as direct repressor of E-cadherin *in vitro* and *in vivo* [37] due to interaction of C-terminal region of Slug with 5-CACCTG-3′ sequence in the E-cadherin promoter [38]. In our studies, the intensity of staining with anti-Slug antibody was similar in control and IFN-modified MM-4 mouse melanoma cells. The same held true for Twist expression except for increasing Twist level in nuclei of MM-4 IFN-beta modified cells.

Recently it was shown that Twist may be involved in suppression of E-cadherin gene and in EMT activation that is relevant to tumor progression [39]. Nevertheless, in our experiments expression of Slug and Twist do not seem to be associated with suppression of MM-4 cell malignancy upon long-term exposure to IFN-beta. This suggests that in these cells expression of Slug and Twist transcription factors does not affect the expression of E-cadherin supposing other mechanisms associated with suppression effect of IFN. These data suggest that IFN-beta can inhibit EMT through some factors other than transcription factors under study.

Moreover, proteins that regulate the expression of adhesion proteins avoiding EMT-associated regulatory elements may be the targets for IFN-beta through the ISRE system. In some studies, the effectiveness of high or low dose IFN-therapy of melanoma is assessed by its duration. In most cases, the duration of therapy does not exceed 1 year, but clinical experience indicates that persistent remission can be prolonged when IFN therapy is extended over several years [40, 41, 42]. Our results obtained *in vitro* clearly speak in favor of long-term effects of IFN therapy.

### 3.7. Tumorigenicity and Metastatic Ability of Melanoma Cells *In Vivo*

In the series of experiments *in vivo*, the effect of long-term impact IFN-beta on tumorigenicity and melanoma growth upon i/m administration of cells was examined. Our studies have shown that modification of MM-4 cells by IFN-beta significantly inhibits kinetics of melanoma growth in mice (Figure **8**). While in the both groups tumor growth was evident in 100 % of animals in 12 days after grafting the cells, in mice injected with MM-4/IFN-b cells noticeable suppression of tumor growth become visible starting from Day 20. Inhibition of tumor growth on Day 25 amounted to 56% as compared to controls. While in mice with grafted MM-4 cells, tumor volume was 3062 ± 354 $mm^3$, whereas in animals grafted with IFN- modified cells — 1422 ± 347 $mm^3$ ($p < 0.01$). These data suggest that MM-4 cells, modified by low dose of IFN-beta possess the decreased growth ability *in vivo*.

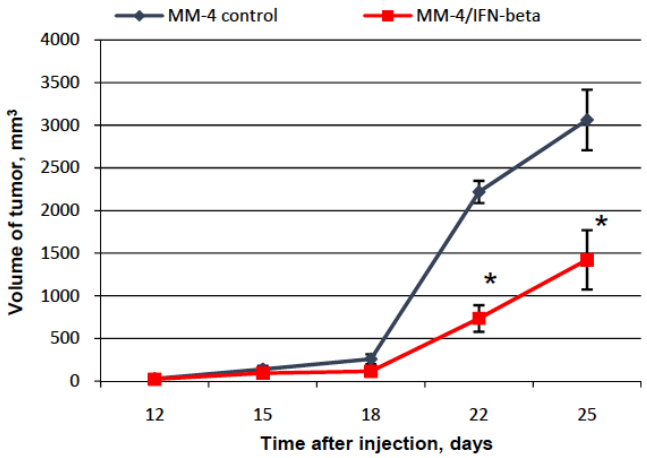


**Figure 8:** Kinetics of melanoma growth in mice after i\m injection of melanoma cells. (*p< 0.01 compared to control) 1. MM-4 control – untreated cells; 2.MM-4/IFN-beta – MM-4 cells, treated by 500 IU/ml IFN-beta for 190 days.

The study showed that the long-term treated of melanoma cells by recombinant IFN-b reduced their metastatic ability, which manifested in 100-fold decrease volume of metastases in 100 times and 10-fold decrease in their number compared to control (Figure **9**).

All these results show the significant suppression of malignant phenotype, proliferation, migration and capacity to autonomous growth in MM-4/IFN-beta cells. The modifying effect of IFN was persisted for at least 2 weeks after withdrawal of IFN suggesting that effects of

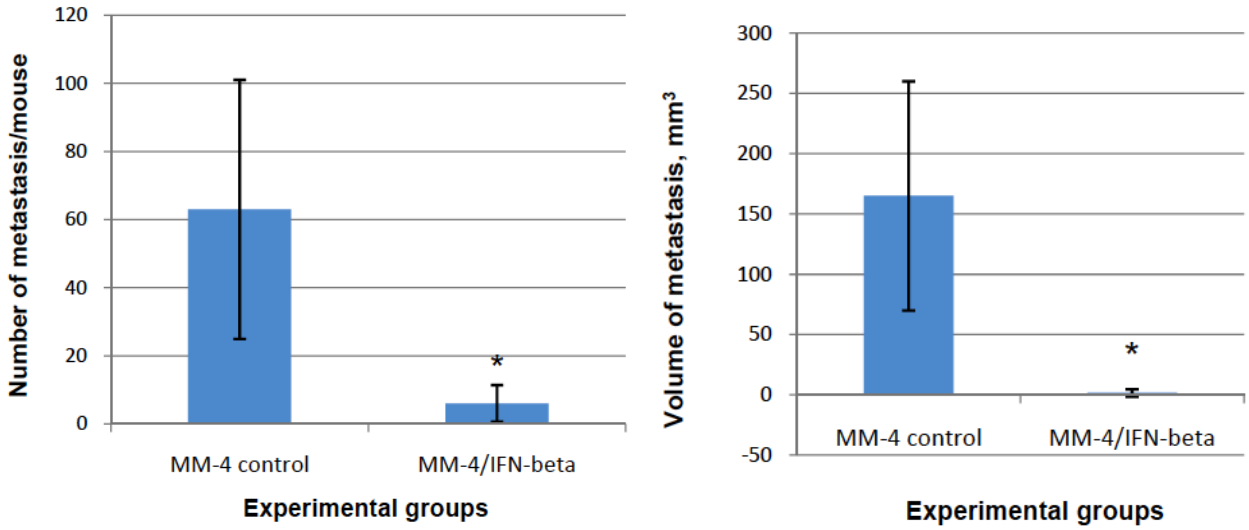


**Figure 9:** Metastatic potential of MM-4 cells. (*p< 0.01 compared to control). 1. MM-4 control– untreated cells, 2. MM-4/IFN – MM-4 cells, treated by 500 IU/ml IFN-beta 190 days.

IFN do not seem to be limited to phenotype modification. Moreover, in experiments *in vivo* we observed a significant inhibition of tumor growth and metastasis even on day 25 after MM-4/IFN cells inoculation.

## 4. CONCLUSIONS

1. Long-term exposure of mouse melanoma cells (MM-4 cell line) to recombinant IFN-beta results in contact inhibition of cell motility, inhibition of cell proliferation and suppression of cell migration.

2. Prolonged culture of MM-4 cells in the presence of IFN-beta inhibits their ability to form colony in semi-solid agar.

3. The long-term treatment of melanoma cells with IFN-beta causes the decrease on the expression of N-cadherin, VE-cadherin and appearance of the nuclear expression of tumor suppressor - p21(Waf1) protein.

4. Modification of MM-4 cells by low dose recombinant IFN-beta inhibits their ability to induce solid tumors and metastases in the lungs of mice.

5. The results suggest the persistent modifying effects of long-term low dose IFN treatment of MM-4 melanoma cells.

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