

ADMINISTRATION OF VITAMIN D3 IMPROVES ANTIMETASTATIC EFFICACY OF CANCER VACCINE THERAPY OF LEWIS LUNG CARCINOMA

E. Zhuravel, O. Efanova, T. Shestakova, N. Glushko, O. Mezhuev, M. Soldatkina, P. Pogrebnoy*

R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv, Ukraine

Aim: To analyze antitumor efficacy of experimental cancer vaccine therapy combined with introduction of vitamin D3 (VD3) for treatment of Lewis lung carcinoma (3LL). **Materials and Methods:** Cancer vaccines composed from recombinant murine beta-defensin-2 (mBD-2) and 3LL cell lysate, or DNA, coding for mBD-2-Muc1 fusion construct cloned in pcDNA3+ vector, were prepared and used for intradermal vaccination. Experimental cancer vaccines introduced i. d. at therapeutic and prophylactic regimens to 3LL-bearing C57Bl mice, were applied alone or in combination with VD3 (administered *per os*) and/or low-dose cyclophosphamide (CP, administered intraperitoneal). Efficacy of treatments was analyzed by primary tumor growth dynamics indexes and by metastasis rate in vaccinated animals. **Results:** As it has been shown, administration of the protein-based vaccine composed from mBD-2 and 3LL cell lysate in combination with VD3 and CP, but not in VD3 free setting, led to significant suppression of primary tumor growth ($p < 0.005$) and had significant antimetastatic effect. Introduction of VD3 with or without CP in the scheme of treatment with mBD-2-Muc1-DNA vaccine at therapeutic regimen has led to significant suppression of primary tumor ($p < 0.05$) and metastasis volumes ($p < 0.005$), while in the groups of animals treated with DNA-vaccine + VD3 with or without CP at prophylactic regimen, significant antimetastatic effect ($p < 0.05$) and elevation of average life-span ($p < 0.05$) have been registered. **Conclusion:** The results of this pilot study have shown promising clinical effects of VD3 administration in combination with cancer vaccinotherapy *in vivo*. **Key Words:** experimental cancer vaccine, murine beta-defensin-2, mucin-1, Lewis lung carcinoma, vitamin D3, cyclophosphamide.

Immunotherapy with the use of rationally designed cancer vaccines is presently considered as a promising approach for cancer treatment directed on enhancement of immune response against specific antigens expressed in tumor cells. As a rule, tumor antigens are poorly immunogenic, that's why vaccination with tumor-associated antigens (TAAs) only doesn't lead to induction of effective antitumor immunity. For generation of effective antitumor response it is considered reasonable to enhance it via introduction of specific chemokine molecules used in vaccine formulation as TAA carriers to antigen-presenting cells [1–3]. In the last years, along with a number of chemokines (CCL7, CCL20, CXCL10 etc.), an antimicrobial peptide murine beta-defensin-2 (mBD-2), a molecule which ability to bind CCR6 on iDCs [4], has been used in experimental cancer immunotherapy as the carrier of genetically fused TAA, what improved significantly the clinical efficacy of the vaccinations [5–7]. In the present research devoted to experimental cancer vaccine therapy, taking into account the data of immunologic studies [5–7], we have chosen mBD-2 as a chemokine in the content of cancer vaccines; from the other side we wish to explore whether activation of *in vivo* expression of this peptide antibiotic may be beneficial for clinical effect of cancer immunotherapy.

mBD-2 belongs to the family of small (2–6 kD) cationic microbicidal peptides that are produced by epithelial cells in response to bacterial products and proinflammatory cytokines, possess multiple biologic

activities, in particular immunomodulatory ones, and compose an important chain of innate immunity system [8, 9]. Moreover, similarly to other mammalian defensins [10–12], mBD-2 may be involved in tumorigenesis playing a complex and poorly understood yet role in cancer cells and tumor microenvironment. According to our recent results [12], mBD-2 expressed in murine Lewis lung carcinoma (3LL) cells, may possibly be involved in regulation of 3LL cell proliferation *in vitro* and *in vivo* playing antiproliferative role in this experimental tumor. Down-regulation of mBD-2 mRNA expression in 3LL cells *in vivo* led to accelerated tumor growth and more aggressive metastasis [12]. Therefore, we hypothesized that up-regulation of mBD-2 expression in epithelial tissues of 3LL-bearing mice in addition to immunotherapy could improve total anticancer effect of the treatment. In this regard we have decided to analyze whether it is possible to activate mBD-2 mRNA expression *in vivo* by introduction of vitamin D3 (VD3) to experimental animals. According to the literature data, some human antimicrobial peptides (cathelicidin [13] and human beta-defensin-2 [14]) are positively regulated by $1.25(\text{OH})_2\text{D}_3$ — metabolic form of VD3. The study [15] performed on mice has shown that mBD-2, mBD3 and cathelin-related antimicrobial peptide are up-regulated in mouse skin exposed to low-dose UV irradiation, and this process is mediated by cutaneous VD3 activation.

To our knowledge, despite wide interest to VD3 as potent pleiotropic immunomodulating agent that may be used alone or in combination with chemotherapy [16] significantly improving anticancer effect of treatment, there are just few publications in the field on its combined use in a setting of cancer immunotherapy. For example, in the work [17], the research has been

Received: February 4, 2010.

*Correspondence: E-mail: pogrebnoy@onconet.kiev.ua

Abbreviations used: 3LL – Lewis lung carcinoma; CP – cyclophosphamide; DC – dendritic cell; mBD-2 – murine beta-defensin-2; Muc1 – murine mucin-1; TAA – tumor-associated antigen; VD3 – vitamin D3.

performed on 3LL model, where the efficacy of applied immunotherapy (adoptively transferred tumor-reactive lymph node cells) has been shown to be significantly elevated by introduction of VD3; the authors have registered the significant antimetastatic effect of such treatment. In a series of works [18–20] it has been demonstrated that VD3/calcitriol may be considered an effective mucosal adjuvant agent potently promoting immune responses to cutaneously administered vaccines.

So, the first task of our work was to study the expression patterns of mBD-2 mRNA in lung tissue of healthy mice treated with VD3, and the second one was to prepare anticancer protein-based and DNA-based vaccines and analyze whether their efficacy *in vivo* in 3LL tumor model may be increased by an introduction of VD3.

MATERIALS AND METHODS

Cell lines and bacterial strains. *In vitro* culture of transplantable 3LL cells was 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 (Kyiv, Ukraine). The cells were cultivated *in vitro* in DMEM culture medium with high glucose content supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B as fungizone in 5% CO₂ atmosphere at 37 °C.

Cultivation of bacterial cells (strain *E. coli* DH5α) was carried out on LB medium at 37 °C. Selection and storage of recombinant colonies was performed on agarized LB medium supplemented with 50 µg/mL ampicilline or kanamycine.

Gene cloning and plasmid construction. The pcDNA3-Igk-mBD-2 expression plasmid containing secretable mBD-2 was created by the following way. Mouse Igk signal sequence was cloned from plasmid pSecTag2a to pcDNA3.1+. Gene for mature mBD2 was obtained from lipopolysaccharide (10 ng/ml)-treated murine BALB/c macrophages by RT-PCR using the following primers: mBD-2-F: 5'-ACCTAAGCTTCGAACCTGACCACTGCCACACC-3' and mBD-2-R: 5'-GCCGAATTCTCATTTTCATGACTTGCAACAGGG-3. The mature mBD2 cDNA was cloned in frame with Igk signal sequence.

For construction of pcDNA3-Igk-mBD-2-Muc1, extracellular domain of Muc1 fragment containing 11 tandem repeats was cloned by RT-PCR from total RNA of 3LL cells with the use of the following PCR primers: Muc1-F: 5'-GCCGAATTCACCAGCAGTTTCCTTAGCATC-3'; Muc1-R: 5'-CTACTCGAGTCATGCAGAGCTGGTAGTTGTGAC-3'; and for mBD-2 fragment: mBD-2-F and CmBD-R: 5'-GGCGAATTC AAGATCGCTTTTCATGACTTGCAACAGGG-3'. The N-terminus of Muc1 was fused in frame with mBD-2 through the linker sequence. The Muc1 fragment for the control vector pcDNA3-Igk-Muc1 was amplified using the following primers pair: CMuc1-F: 5'-AACAAGCTTCAC-CAGCAGTTTCCTTAGCATC -3'; Muc1-R.

All constructs were verified by DNA dideoxysequencing method using T7 and BGH primers and purified using plasmid purification kit Quiagen EndoFree (USA). Content of LPS in plasmid preparation and protein-based vaccine was evaluated by standard LAL-test.

Control transfection of human embryonal kidney (HEK293) cells was performed with the use of FuGene 6 reagent (Roche Molecular Biochemicals, USA) according to the instructions of the manufacturer.

RT-PCR analysis. Total RNA was isolated from tissue samples by the method of Chromzynski and Sacchi [21]. For detection of mBD-2 or Muc1 RNA expression, semiquantitative RT-PCR analysis was performed with the use of specific primers. The expression level of beta-actin as the house-keeping gene served as a control. The relative expression level was analyzed with the use of TotalLab Program.

Preparation of protein-based vaccine. For the preparation of protein-based vaccine, active recombinant mBD-2 produced in bacterial cells as GST-fusion protein [12], that was additionally purified from endotoxin contaminants has been used. 3LL cell lysate was prepared from *in vitro* cultured 3LL cells by standard lysis of the cells with modified SDS-free RIPA buffer, and stored at -70 °C until use.

In vivo study. For *in vivo* research, male C57Bl mice 2 months old bred in the animal facility of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine) were used. All animal procedures were carried out according to the rules of local Ethic Committee and were approved by the Ethic Board of IEPOR NASU.

3LL cells (5 × 10⁴ cells/100 µl PBS per animal) were transplanted i.m. in right hind leg of C57Bl mice. Tumor growth dynamics was monitored each 5 days by means of calipers starting from day 17 after tumor cell transplantation when tumors became palpable, and till the end of experiment. At 34-th day after tumor cell transplantation, the animals were sacrificed by ether narcosis, primary tumors and lungs were removed and weighted, lung metastases were calculated, and blood serum was collected.

Immunotherapy of experimental murine tumors with the use of cancer DNA vaccine or protein-based vaccine was performed at therapeutic and prophylactic regimens by similar schedules. In therapeutic setting, vaccine was introduced i.d. at days 2, 6, 10, and 20 after tumor cell transplantation [5]; in prophylactic one — at days 0, 4, 8, 18; in 2 weeks after the last immunization, 3LL cells were transplanted. For i.d. administration of DNA vaccine, the first three immunizations (20 µg DNA/10 µL PBS) were performed with the use of tattoo device in the skin of left hind leg by the method described in [22], while the fourth vaccination (20 µg DNA/10 µL PBS) was done i. d. in the ear of animals. In the case of protein-based cancer vaccine, it was injected i. d. only in the ear of animals (recombinant mBD-2 peptide (2 µg/10 µl PBS) and 3LL cell lysate (20 µg/20 µl PBS)).

VD3 (10 IU/day/animal) was administered with drinking water. Cyclophosphamide (CP, 2.5 mg/100 μ L PBS/animal) was administered i. p. at the days 1, 9, 19 after tumor cell transplantation as described in [23].

Experimental animals were housed in 10 groups per each protein-based vaccination setting (A — therapeutic regimen, B — prophylactic regimen; n = 5 per group) and received the following treatment: A1 (B1) — control; A2 — mBD-2; A3 — 3LL cell lysate; A4 — 3LL + VD3; A5 — 3LL + D3 + CP; A6 — mBD-2 + 3LL; A7 — VD3; A8 — CP; A9 — mBD-2 + 3LL + VD3; A10 — mBD-2 + 3LL + VD3 + CP.

Similarly, for DNA-vaccination there were 10 groups for therapy (C) and 10 groups for prophylaxis (D) (n = 5 per group) that received complete or incomplete plasmid constructs: C1 (D1) — control (blank vector); C2 (D2) — pcDNA3-Igk-mBD-2; C3 (D3) — pcDNA3-Igk-Muc1; C4 (D4) — pcDNA3-Igk-Muc1 + VD3; C5 (D5) — pcDNA3-Igk-Muc1 + VD3 + CP; C6 (D6) — pcDNA3-Igk-mBD-2-Muc1; C7 (D7) — VD3; C8 (D8) — CP; C9 (D9) — pcDNA3-Igk-Muc1 + VD3; C10 (D10) — pcDNA3-Igk-mBD-2-Muc1 + VD3 + CP.

Antitumor and antimetastatic effects of vaccination were evaluated by suppression of primary tumor growth and the number of lung metastases. Each experiment was repeated twice.

Statistical analysis. The data were reported as the mean \pm SD. The statistical significance of the differences between mean values was assessed by the Student's *t*-test. Values $p < 0.05$ were considered statistically significant. Differences in survival between the groups were determined by nonparametric log-rank test.

RESULTS AND DISCUSSION

VD3 treatment up-regulates mBD-2 mRNA expression in murine lung tissue. Prior to vaccinations, we have performed an *in vivo* study in order to analyze whether it is possible to activate mBD-2 mRNA expression *in vivo* in epithelial lung cells of healthy mice by introduction of VD3. Experiment has been carried out on healthy C57Bl male mice (n = 3 per group) that received VD3 (10 IU per animal per day) *per os* for 10 days, or did not receive the vitamin. Then the animals were sacrificed, and mBD-2 mRNA expression level in healthy lung tissues of the animals was analyzed with the use of semiquantitative RT-PCR analysis. Our data have demonstrated (Fig. 1) that consumption of VD3 resulted in up-regulation of mBD-2 expression in healthy lungs of C57Bl mice compared to the control. So, we have expected that introduction of VD3 in immunotherapy schedule may lead to up-regulation of mBD-2 expression in lung tissues of experimental animals and, respectively, to elevation of its local antiproliferative activity [12] what may possibly improve the results of experimental immunotherapy.

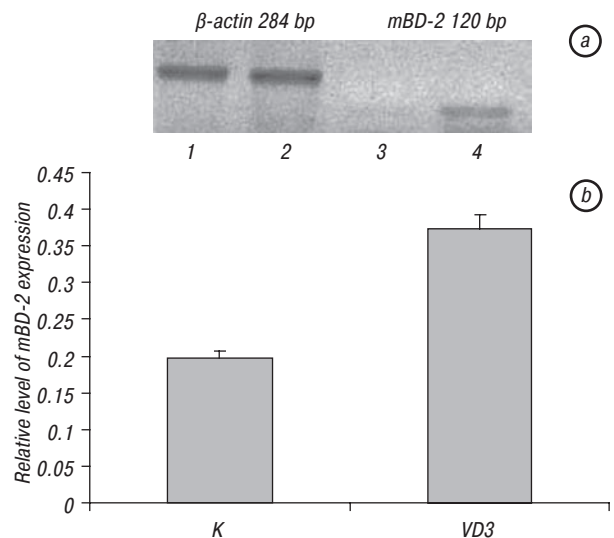


Fig. 1. RT-PCR analysis of mBD-2 mRNA expression in lung tissue of healthy mice (a): mBD-2 mRNA expression in lungs of healthy animals that were treated with VD3 (line 4) vs that in control animals (line 3). Graphical representation of mBD2 mRNA expression level normalized by beta-actin expression (b)

Design of experiment and selection of tumor model. For performance of vaccinations, we have used 3LL, an experimental model of murine solid tumor with high level metastasis in lungs. Transplantation of just 5×10^4 3LL cells/animal results in 100% lethality in 5 weeks period.

In a setting of protein-based vaccine, we have used 3LL cell lysate as total tumor antigen.

In a setting of DNA-based vaccine, to select a protein target in 3LL cells, we have performed an analysis of literature data in the field, and decided to introduce murine mucin-1 (Muc1) coding sequence in DNA vaccine. Muc1 is a highly glycosylated 200 kDa membrane protein [24], a non-polymorphic homolog of human polymorphic epithelial mucin MUC1. The human MUC1 is expressed by a wide variety of epithelial tissues where the protein plays the important physiologic and antiinflammatory roles at normal state [25, 26]. In human epithelial cancers, MUC1 is often overexpressed, underglycosylated and lost polarized type of expression, that's why it is recognized as universal tumor antigen of human carcinoma. There are numerous experimental researches of cancer vaccines directed against MUC1 performed on MUC1-transgenic animals, and clinical trials of cancer vaccines directed against MUC1 [27, 28]; and their results are promising. In contrary, little is known about expression patterns of Muc1 in murine tumors, and experimental vaccinations with the use of wtMuc1 have been done rarely. According to the data of literature [29], earlier with the use of experimental breast cancer model there were performed immunizations in C3H/HeOuj mice with mouse mucin-1 fusion protein, and in a case of combination of such immunotherapy with CP treatment antitumor effect has been registered. As far as we did not found the data on Muc1 expression patterns in murine tumor cells and in particular, in 3LL cells, we have performed semiquantitative RT-PCR analysis of Muc1 mRNA expression in 3LL-derived tumors, healthy lungs and

heavily metastasized lungs of 3LL-bearing mice. We have registered high expression levels of Muc1 mRNA both in 3LL derived tumors, metastatic lesions, and in healthy lung cells of C57Bl mice (Fig. 2); so, being certain that Muc1 is expressed in 3LL cells, we have supposed to direct our vaccine against Muc1 in this particular tumor model despite the understandable risk of its low specificity and autoimmunity.

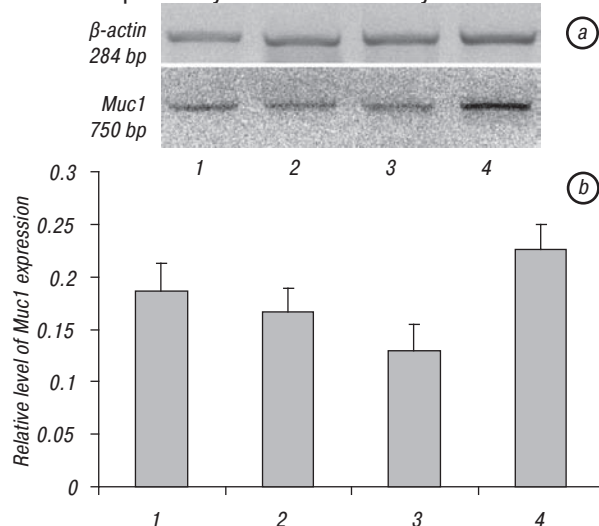


Fig. 2. RT-PCR analysis of Muc1 mRNA expression in largely metastasized murine lung tissue (line 4), healthy lung tissue (lines 1, 2) and primary 3LL tumor sample (line 3). Expression of beta-actin served as a control (a). Graphical representation of mBD2 mRNA expression level normalized by beta-actin expression (b)

At last, according to literature data [23, 30], cancer immunotherapy efficacy could be elevated by its combination with low-dose chemotherapy for inhibition of immunosuppressive cancer network. That's why we have supplemented the vaccination schedule with low-dose CP therapy (100 mg/kg).

Vaccination of 3LL-bearing mice with cancer vaccine composed from recombinant mBD-2 peptide and 3LL cell lysate. To analyze antitumor effect of vaccination with the use of cancer vaccine composed from recombinant mBD-2 peptide (2 µg/10 µl PBS) and 3LL cell lysate (20 µg/20 µl PBS), we have applied both therapeutic and prophylactic administration of the vaccine and its separate components with or without VD3 and CP therapy by the schemes described in Materials and Methods section.

The results of therapeutic vaccination have shown (Fig. 3, 4) that administration of mBD-2 peptide together with 3LL cell lysate led to statistically insignificant suppression of primary tumor growth and metastatic levels in 3LL-bearing mice compared with control groups. However, introduction of VD3 into treatment schedule, especially in combination with CP therapy at metronomic regimen, has notably affected an efficacy of such vaccination and resulted in significant decrease of primary tumor volumes (group A10, $p < 0.005$, see Fig. 3) and the number of lung metastases (see Fig. 4). Interestingly, similar antimetastatic effect of VD3 administration has been observed in a control group of animals treated with 3LL cell lysate + VD3 (see Fig. 4)

but not in the case of separate VD3 or 3LL cell lysate administration, and there were insignificant differences in lung metastasis numbers between 3LL + VD3 and mBD-2 + 3LL + VD3 treated groups. Administration of CP only didn't influence significantly primary tumor volumes, but had significant antimetastatic effect (group A8). Upon prophylactic administration of the vaccine, also the highest antimetastatic effect has been observed in the case of administration of the vaccine simultaneously with VD3 consumption (mean lung weight in vaccine + VD3 treated animals was 190 ± 15 mg vs 295 ± 145 mg in control groups). Both prophylactic and therapeutic vaccinations had statistically insignificant influence on average life span of tumor-bearing mice (Fig. 5).

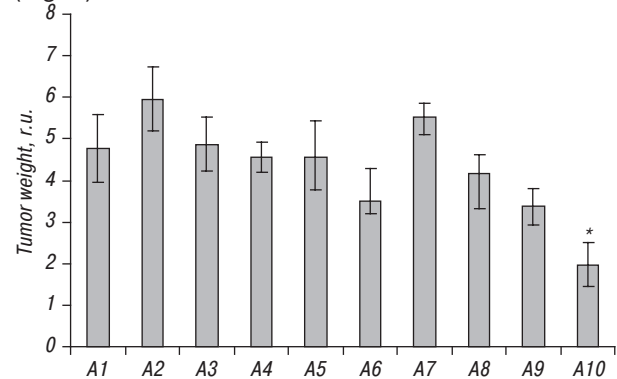


Fig. 3. Comparative analysis of primary tumor weight (rel. units) in experimental animals from groups A1–A10 treated with protein-based vaccine at therapeutic regimen at the day 34 after 3LL transplantation: A1 — control; A2 — mBD-2; A3 — 3LL cell lysate; A4 — 3LL + VD3; A5 — 3LL + D3 + CP; A6 — mBD-2 + 3LL; A7 — VD3; A8 — CP; A9 — mBD-2 + 3LL + VD3; A10 — mBD-2 + 3LL + VD3 + CP. Each group was composed from 5 animals, experiment was twice repeated. *Difference is significant ($p < 0.005$) compared to group A1.

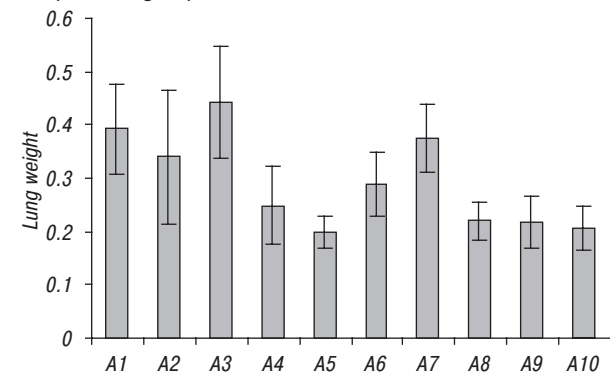


Fig. 4. Lung weight of experimental animals from groups A1–A10 treated with protein-based vaccine at therapeutic regimen at the day 34 after 3LL transplantation. Treatment groups: A1 — control; A2 — mBD-2; A3 — 3LL cell lysate; A4 — 3LL + VD3; A5 — 3LL + D3 + CP; A6 — mBD-2 + 3LL; A7 — VD3; A8 — CP; A9 — mBD-2 + 3LL + VD3; A10 — mBD-2 + 3LL + VD3 + CP. Each group was composed from 5 animals, experiment was twice repeated

So, from the data of mentioned above experiments we have figure out two main conclusions. Firstly, an anticancer efficacy of protein [mBD-2 + 3LL lysate] vaccination is moderate enough and did not generate significant therapeutic and protective immunity in 3LL model. Such results could be explained by the data of investigation [4] where the authors have shown that

effective vaccinations using beta-defensin-2 require an existence of physical link between peptide and TAA to elicit antitumor immunity. Possibly, an absence of chemical coupling between recombinant mBD-2 peptide and TAAs in 3LL cell lysate may explain moderately good results of vaccination of 3LL bearing mice. Secondly, our results have demonstrated that introduction of VD3 into the scheme of cancer immunotherapy may be considered reasonable and, without being toxic, notably elevates antimetastatic effect of vaccination, even in its defensin-free setting (see Fig. 4). Besides, the use of VD3 alone also led to some decrease of secondary tumors volumes, however such rates were statistically insignificant; it couldn't be excluded that mentioned effect may be in part realized via up-regulation of *mBD-2* gene in murine epithelial tissues. Both mentioned conclusions we have taken into account in the second part of our research directed on generation of mBD-2-containing cancer DNA vaccine.

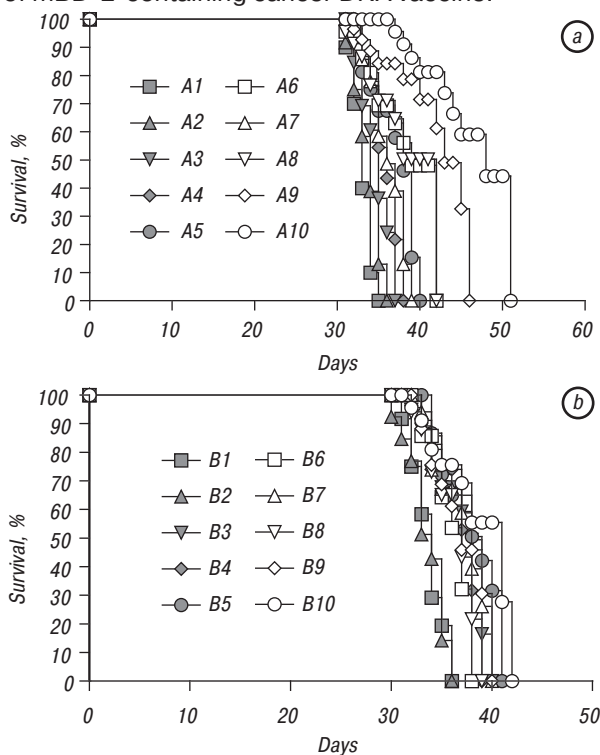


Fig. 5. Survival of 3LL-bearing mice treated with protein-based vaccine at therapeutic (a) and prophylactic (b) regimens. Each group of animals was composed from 10 animals. Log-rank test. Treatment groups: A1 (B1) — control; A2 (B2) — mBD-2; A3 (B3) — 3LL cell lysate; A4 (B4) — 3LL + VD3; A5 (B5) — 3LL + D3 + CP; A6 (B6) — mBD-2 + 3LL; A7 (B7) — VD3; A8 (B8) — CP; A9 (B9) — mBD-2 + 3LL + VD3; A10 (B10) — mBD-2 + 3LL + VD3 + CP. *Difference is significant ($p < 0.05$) compared to the control groups treated with blank vector.

Construction of cancer DNA vaccine composed from mBD-2 and murine Muc1. To generate cancer DNA vaccine composed from mBD-2 and murine Muc1, we have constructed DNA vector — chimeric molecule containing the coding sequences of mature *mBD-2* and murine *Muc1* genes by the procedure described in Materials and Methods section.

The constructs were tested by control transfection of human embryonal kidney HEK293 cells, with the next RT-PCR analysis of Muc1 mRNA expres-

sion (Fig. 6). RT-PCR analysis has demonstrated that transfection was effective, and the transfected HEK293 cells express Muc1 mRNA (see Fig. 6).

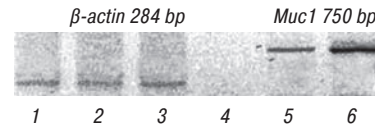


Fig. 6. RT-PCR analysis of Muc1 mRNA expression in control HEK 293 cells (4) and transfected HEK-pcDNA3-Igk-Muc1 (5) and HEK-pcDNA3-Igk-mBD-2-Muc1 cells (6). Lines 1–3 — expression of beta-actin gene in HEK 293, HEK-pcDNA3Igk-Muc1 and HEK-pcDNA3Igk-mBD-2-Muc1 cells served as a control

So, in following research we have used the next panel of vector constructs: pcDNA3.1+, pcDNA3-Igk-mBD-2, pcDNA3-Igk-Muc1, pcDNA3-Igk-mBD-2-Muc1.

Evaluation of anticancer efficacy of mBD-2-Muc1-cancer DNA vaccine. To evaluate anticancer efficacy of the developed mBD-2-Muc1-DNA vaccine, we have apply vaccination protocol consisting from four sequential intradermal immunizations (20 μ g DNA/10 μ l PBS per animal) — first three with the use of tattoo device in skin of animal's leg, and the fourth one — i. d. in ears of mice, at therapeutic (series C) and prophylactic (series D) regimens, as described in Materials & Methods section.

As it has been mentioned above, transplantation of 5×10^4 3LL cells/animal results in 100% mortality in 5 weeks period; as we have found out, vaccination with the use of DNA vaccine + VD3 + CP led to generation of tumor protection in 20% of mice that received immunotherapy at prophylactic and therapeutic regimens. In the rest of animals that developed the tumors, therapeutic vaccination suppressed significantly primary tumors growth ($p < 0.05$) (Fig. 7) and led to significant reduction of metastasis ($p < 0.005$) in animals treated with vaccine + VD3 and with/without CP (Fig. 8). Prophylactic vaccination led to statistically insignificant suppression of primary tumor growth in animals treated with vaccine + VD3 + CP and significant decrease of metastasis rate in groups D4, D9, D10 (see Fig. 8); in groups C9, C10, D9 and D10 (pcDNA3-Igk-mBD-2-Muc1 + VD3 with or without CP) we have registered also the significant increase of average life-span of tumor-bearing animals ($p < 0.05$, Fig. 9).

In conclusion, the data of *in vivo* experiments evidence on positive clinical effect of VD3 introduction into scheme of mBD-2-Muc1-DNA-based vaccination combined with low-dose CP treatment, reflected in significant antimetastatic effect and increase of average life-span of tumor-bearing animals. To be explained, the aforementioned results, without a doubt, require immunological studies, and we'll perform them in our further research.

According to recent knowledge, VD3 is a pleiotropic potent regulator of mammalian immune system [30]. Its ability to suppress autoimmune reactivity is demonstrated in experimental models of type 1 diabetes, systemic lupus erythematosus, inflammatory bowel disease, autoimmune thyroiditis etc. [31, 32]. Along with immunosuppressive activity, paradoxically, VD3 has a

positive effect as well on the system of innate immunity enhancing its response against invading microorganisms; VD3 deficiency is believed to be associated with susceptibility to some infectious diseases, in particular, tuberculosis [33]. In the last case, some possible mechanisms of innate defense against *Mycobacterium tuberculosis* are supposed to be realized via VD3-dependent up-regulation of cathelicidin in host macrophages [34]. We believe that in present research the favoring effect of VD3 on antimetastatic efficacy of experimental cancer vaccines possibly may be in some part mediated by VD3-dependent up-regulation of mBD-2 in lung epithelial cells. Meanwhile, recent publications have shown that antitumor activity of VD3 and $1.25(\text{OH})_2\text{D}_3$ *in vivo*, due to multiple target genes of the vitamin, could be realized by many ways including induction of cancer cell apoptosis, regulation of the cell cycle, up-regulation of VD3-receptor (VDR) playing a role in p53-mediated suppression of tumor growth, or regulation of a balance between proangiogenic and antiangiogenic factors thus influencing tumor angiogenesis [35–37]. Further studies in this field will help us understand the mechanisms of immunomodulatory effects of VD3 as potentially effective adjuvant in cancer immunotherapy, and of its direct antitumor action.

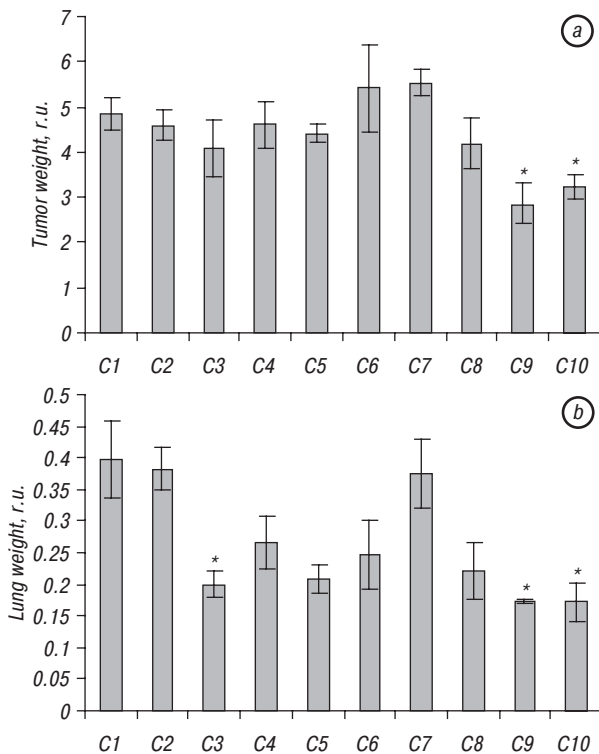


Fig. 7. Primary tumors volume (a) and lung weight (b) in 3LL-bearing mice, treated with DNA vaccine at therapeutic regimen at the day 34 after 3LL transplantation: C1 — control (blank vector); C2 — pcDNA3-Igk-mBD-2; C3 — pcDNA3-Igk-Muc1; C4 — pcDNA3-Igk-Muc1 + VD3; C5 — pcDNA3-Igk-Muc1 + VD3 + CP; C6 — pcDNA3-Igk-mBD-2-Muc1; C7 — VD3; C8 — CP; C9 — pcDNA3-Igk-mBD-2-Muc1 + VD3; C10 — pcDNA3-Igk-mBD-2-Muc1 + VD3 + CP. *a* — *Difference is significant ($p < 0.05$) compared to group C1. *b* — *Difference is significant ($p < 0.005$) compared to group C1. Each group of animals was composed from 5 animals, experiment was twice repeated.

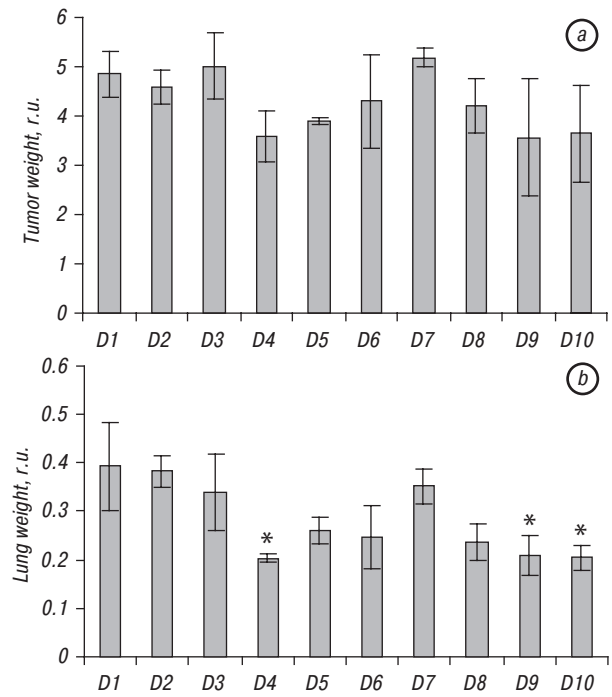


Fig. 8. Primary tumor volume (a) and lung weight (b) in 3LL-bearing mice, treated at prophylactic regimen with DNA vaccine at the day 34 after 3LL transplantation: D1 — control (blank vector); D2 — pcDNA3-Igk-mBD-2; D3 — pcDNA3-Igk-Muc1; D4 — pcDNA3-Igk-Muc1 + VD3; D5 — pcDNA3-Igk-Muc1 + VD3 + CP; D6 — pcDNA3-Igk-mBD-2-Muc1; D7 — VD3; D8 — CP; D9 — pcDNA3-Igk-mBD-2-Muc1 + VD3; D10 — pcDNA3-Igk-mBD-2-Muc1 + VD3 + CP. *Difference is significant ($p < 0.05$) compared to group D1. Each group of animals was composed from 5 animals, experiment was twice repeated.

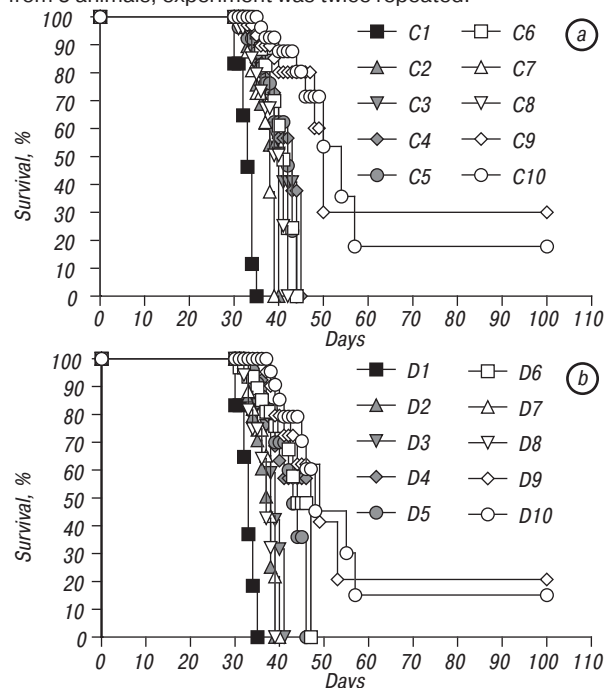


Fig. 9. Survival of 3LL-bearing mice treated with DNA vaccine at therapeutic (a) and prophylactic (b) regimens. Each group of animals was composed from 10 animals. Log-rank test. C1 (D1) — control (blank vector); C2 (D2) — pcDNA3-Igk-mBD-2; C3 (D3) — pcDNA3-Igk-Muc1; C4 (D4) — pcDNA3-Igk-Muc1 + VD3; C5 (D5) — pcDNA3-Igk-Muc1 + VD3 + CP; C6 (D6) — pcDNA3-Igk-mBD-2-Muc1; C7 (D7) — VD3; C8 (D8) — CP; C9 (D9) — pcDNA3-Igk-mBD-2-Muc1 + VD3; C10 (D10) — pcDNA3-Igk-mBD-2-Muc1 + VD3 + CP. *Difference between groups C9 vs C1, and D9 vs D1 are significant ($p < 0.05$).

ACKNOWLEDGEMENTS

The work was supported by NASU grants 0107U005545, the Program “Newest Medico-Biological Problems and Environment”, Part 2. “Biologically Active Compounds for Human Health” (Ukraine), and U002243 “Fundamental Problems of Genomics and Proteomics”.

REFERENCES

- Dell'Agnola C. Clinical utilization of chemokines to combat cancer: the double-edged sword. *Expert Rev Vaccines* 2007; **6**: 287–93.
- Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; **4**: 499–511.
- Jiang ZH, Koganty RR. Synthetic vaccines: the role of adjuvants in immune targeting. *Curr Med Chem* 2003; **10**: 1423–39.
- Yang D, Chertov O, Bikovskaya SN, *et al.* Beta-defensins: linking innate and adaptive immunity through dendritic and T-cell CCR6. *Science* 1999; **286**: 525–8.
- Biragyn A, Belyakov IM, Chow Y, *et al.* DNA vaccines encoding human immunodeficiency virus-1 glycoprotein 120 fusions with proinflammatory chemoattractants induce systemic and mucosal immune responses. *Blood* 2002; **100**: 1153–9.
- Biragyn A, Ruffini PA, Coscia M, *et al.* Chemokine receptor-mediated delivery directs self-tumor antigen efficiently into the class II processing pathway *in vitro* and induces protective immunity *in vivo*. *Blood* 2004; **104**: 1961–9.
- Biragyn A, Surenhu M, Yang D, *et al.* Mediators of innate immunity that target immature, but not mature, dendritic cells induce antitumor immunity when genetically fused with nonimmunogenic tumor antigens. *J Immunol* 2001; **167**: 6644–53.
- Bowdish DME, Davidson DJ, Hancock RE. Immunomodulatory properties of defensins and cathelicidins. *Curr Top Microbiol Immunol* 2006; **306**: 27–66.
- Lehrer RI, Ganz T. Defensins of vertebrate animals. *Curr Opin Immunol* 2002; **14**: 96–102.
- Droina N, Hendrab J-B, Ducoroy P, *et al.* Human defensins as cancer biomarkers and antitumor molecules. *J Proteomics* 2009; **72**: 918–27.
- Coffelt SB, Scandurro AB. Tumor sound the alarmin(s). *Cancer Res* 2008; **68**: 6482–5.
- Zhuravel E, Shestakova T, Glushko N, *et al.* Expression patterns of murine beta-defensin-2 mRNA in Lewis lung carcinoma cells *in vitro* and *in vivo*. *Exp Oncol* 2008; **30**: 206–11.
- Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D₃. *FASEB J* 2005; **19**: 1067–77.
- Wang TT, Nestel FP, Bourdeau V, *et al.* Cutting edge: 1,25-dihydroxyvitamin D₃ is a direct inducer of antimicrobial peptide gene expression. *J Immunol* 2004; **173**: 2909–12.
- Hong SP, Kim MJ, Jung MY, *et al.* Biopositive effects of low-dose UVB on epidermis: coordinate upregulation of antimicrobial peptides and permeability barrier reinforcement. *J Invest Dermatol* 2008; **128**: 2880–7.
- Trump DL, Hershberger PA, Bernardi RJ, *et al.* Antitumor activity of calcitriol: pre-clinical and clinical studies. *Steroid Biochem Mol Biol* 2004; **89–90**: 519–26.
- Wiers KM, Lathers DM, Wright MA, *et al.* Vitamin D₃ treatment to diminish the levels of immune suppressive CD34⁺ cells increases the effectiveness of adoptive immunotherapy. *J Immunother* 2000; **23**: 115–24.
- Enioutina EY, Visic D, McGee ZA, *et al.* The induction of systemic and mucosal immune responses following the subcutaneous immunization of mature adult mice: characterization of in mucosal secretions in animals immunized with antigen formulations containing a vitamin D₃ adjuvant. *Vaccine* 1999; **17**: 3050–64.
- Enioutina EY, Bareyan D, Daynes RA. Vitamin D₃-mediated alterations in myeloid dendritic cell trafficking *in vivo* expand the scope of their antigen presenting properties. *Vaccine* 2007; **25**: 1236–49.
- Enioutina EY, Bareyan D, Daynes RA. TLR ligands that stimulate the metabolism vitamin D₃ in activated murine dendritic cells can function as effective mucosal adjuvants to subcutaneously administered vaccines. *Vaccine* 2008; **26**: 601–13.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–9.
- Bins AD, Jorritsma A, Wolkers MC, *et al.* A rapid and potent DNA vaccination strategy defined by *in vivo* monitoring of antigen expression. *Nat Med* 2005; **11**: 899–904.
- Berraondo P, Nouze C, Preville X, *et al.* Eradication of large tumors in mice by a tritherapy targeting the innate, adaptive and regulatory components of the immune system. *Cancer Res* 2007; **67**: 8847–55.
- Spicer AP, Parry G, Patton S, *et al.* Molecular cloning and analysis of the mouse homologue of the tumor-associated mucin, MUC1 reveals conservation of potential O-glycosylation sites, transmembrane, and cytoplasmic domains and a loss of minisatellite-like polymorphism. *J Biol Chem* 1991; **266**: 15099–109.
- Hatrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol* 2008; **70**: 431–57.
- Kim KC, Lillehoj EP. MUC1 mucin: a peacemaker in the lung. *Am J Respir Cell Mol Biol* 2008; **39**: 644–7.
- Acres B, Limacher JM. MUC1 as a target antigen for cancer immunotherapy. *Expert Rev Vaccines* 2005; **4**: 493–502.
- Tang CK, Katsara M, Apostolopoulos V. Strategies used for MUC1 immunotherapy: human clinical studies. *Expert Rev Vaccines* 2008; **7**: 963–75.
- Pei Xiang X, Poulos G, Mckenzie IF. Breast cancer in mice: effect of murine Muc1 immunization on tumor incidence in C3H/HeOuj mice. *J Immunother* 2001; **24**: 10–8.
- Matsushita N, Pilon-Thomas SA, Martin LM, *et al.* Comparative methodologies of regulatory T cell depletion in a murine melanoma model. *J Immunol Methods* 2008; **333**: 167–79.
- Van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D₃: basic concepts. *J Steroid Biochem Mol Biol* 2005; **97**: 93–101.
- Cantorna MT, Zhu Y, Froicu M, *et al.* Vitamin D status, 1,25-dihydroxyvitamin D₃, and the immune system. *Am J Clin Nutr* 2004; **80**: 1717–20.
- Adams JS. Vitamin D₃ as a defensin. *J Musculoskelet Neuronal Interact* 2006; **6**: 344–6.
- Liu PT, Stenger S, Li H, *et al.* Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006; **311**: 1770–3.
- Diaz GD, Paraskeva C, Thomas MG, *et al.* Apoptosis is induced by the active metabolite of vitamin D₃ and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. *Cancer Res* 2000; **60**: 2304–12.
- Maruyama R, Aoki F, Toyota M, *et al.* Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. *Cancer Res* 2006; **66**: 4574–83.
- Fernandez-Garcia NI, Palmer HG, Garcia M, *et al.* 1alpha,25-Dihydroxyvitamin D₃ regulates the expression of Id1 and Id2 genes and the angiogenic phenotype of human colon carcinoma cells. *Oncogene* 2005; **24**: 6533–44.