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DOWN-REGULATION OF MURINE BETA-DEFENSIN-2 IN LEWIS LUNG CARCINOMA CELLS RESULTS IN ACCELERATED GROWTH OF TUMOR CELLS *IN VITRO* AND *IN VIVO*

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Aim: **To evaluate the anti-tumor activity of murine beta-defensin-2 (mBD-2) expression *in vitro* and *in vivo*. *Materials and Methods:* Based on pcDNA3 vector, constructs containing mBD-2 cDNA coding mature defensin molecule (pcDNA3-mBD2), and Igk-mBD-2 insertion, coding secretory sequence plus mature defensin molecule (pcDNA3-Igk-mBD-2) were generated. Lewis lung carcinoma (3LL) cells were transfected *in vitro* with these plasmids and with blank pcDNA3 vector, and the proliferative rate and clonogenic ability of obtained cell lines cultivated *in vitro* were analyzed using ^{3}H-incorporation technique and colony formation in semi-soft medium, respectively. Expression of mBD-2 mRNA was studied by semiquantative RT-PCR analysis. Also, transfected cells were transplanted to C57B mice, and the patterns of tumor growth *in vivo* were analyzed by routine techniques. *Results:* We have found out that in the 3LL cells transfected with pcDNA3-mBD-2 and pcDNA3-Igk-mBD-2, the expression of mBD-2 mRNA is significantly down regulated compared to wild-type cells and 3LL cells transfected with blank vector. The cells with suppressed mBD-2 expression differed from parental cells and cells transfected with blank vector by higher proliferation rate ($p < 0.001$) and higher clonogenic ability. The 3LL-mBD-2 and 3LL-Igk-mBD-2 cells that are transplanted to C57B mice gave rise to more aggressive tumors that possessed significantly higher growth rate ($p < 0.01$) than those that arise from wild-type 3LL cells. *Conclusion:* The obtained results are evidencing on a possible tumor-suppressing role of mBD-2 expression.**
Key Words: **murine beta-defensin, Lewis lung carcinoma, expression, proliferation, colony formation.**

Defensins, mammalian antimicrobial peptides with pluripotent biological activities, are a family of cationic cysteine-rich peptides which spectrum of action largely depends on their local concentration and microenvironment [1]. By the differences in their tertiary structure and disulfide bonding, defensins are distributed in three families, namely, α-, β- and θ-defensin; the places of their expression in the body are different: while α-defensins are expressed by netrophils/leucocytes, β-defensins are found mainly in epithelial cells.

Recently these molecules are recognized also as the possible members of antitumor defense arsenal due to their ability to cause lyzis of tumor cells [2], and immunomodulatory and chemokine activities [3–6], in particular, ability to attract immature dendritic cells. This important pattern of beta-defensins has been tested in few experimental studies, where defensin gene was introduced to tumor cells that lack its expression, and its *in vivo* expression resulted in suppression of tumor growth *in vivo* [7, 8]. From the other hand, defensins may have positive or negative impact on tumor growth due to their proangiogenic properties (murine beta-defensin-29 [9]) or antiangiogenic activities (HNP-1 and HNP-3 [10]). Moreover, there is a number of reports where pro-proliferative action of defensins *in vitro* [11-13] has been demonstrated, or, in contrary, no effect of hBD-2 on proliferation has been stated [14]. So, the question on possible role of defensins in tumorigenesis remains largely unanswered.

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Abbreviations used: 3LL – Lewis lung carcinoma; mBD-2 – murine beta-defensin-2.

The present research continues our studies of the functional role of beta-defensin expression in cancer cells. For the study, Lewis lung carcinoma (3LL) model was chosen. 3LL is an experimental murine tumor model widely used in cancer research. 3LL cells are expressing murine beta-defensin-2 (mBD-2), the homolog of human beta-defensin-1, at a constant moderate level. We have introduced an additional copy of *mBD-2* gene in 3LL cells with the aim to receive its hyperexpression to achieve possible suppression of 3LL growth *in vivo*. However, after such manipulation we have received an opposite effect: surprisingly, transfection of 3LL cells with pcDNA-mBD-2 and pcDNA-Igk-mBD-2 vectors resulted in down-regulation of mBD-2 mRNA expression in these cells in parallel with acquired higher proliferative rate and colony forming ability *in vitro*, and higher aggressiveness of experimental tumors that arised from the cells with suppressed mBD-2 expression. Taken together, such results are pointing on a possible tumor-suppressor role of mBD-2.

MATERIALS AND METHODS

Cell lines and bacterial strains. *In vitro* culture of Lewis lung carcinoma (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 G sodium, 100 μg/mL streptomycin sulfate and 0.25 μg/mL amphotericin B as fungizone in 5% CO_2 athmosphere at 37 °C.

For testing the antimicrobial activity of recombinant mBD-2 in the test of Hultmark et al. [15], *Pseudomonas*

aeruginosa ATCC 9027 was used; the strain was kindly gifted by Dr. E. Kiprianova from the D.K. Zabolotny Institute of Microbiology and Virology NAS of Ukraine (Kyiv, Ukraine).

Generation and purification of recombinant mBD-2. To clone the gene coding mature mBD-2 molecule, mBD-2 cDNA was received by RT-PCR with the use of specific primers (mBD-2-F: 5`-ACCG-GATCCACCATGGAA CTTGACCACTGCCACACC-3` mBD-2-R: 5`-GCCGAATTCTCATTTCAT GTACTTG-CAACAGGG-3`) from total RNA isolated from murine BALB/c macrophages incubated for 24 h with 10 ng/ml lipopolysaccharides of *P. aeruginosa*. Primers were designed on the base of AJ011800 sequence from GenBank database.

For production of recombinant mBD-2, we have chosen the GST-expression system, that allows the production of recombinant proteins fused with glutathione-S-transferase (GST) in bacterial cells upon induction of isopropyl-β-D-thiogalactopiranoside (IPTG). Respectively, mBD-2 cDNA fragment was cloned in pGEX-2T vector by *BamHI* and *EcoRI* sites using routine procedure. PCR product and pGEX-2T vector were purified, treated with restriction endonucleases *BamHI* and *EcoRI*, ligated by routine technique, and correctness of insertion was verified by the DNA dideoxy sequencing method and restriction analyzis. Then *E. coli BL21DE3* cells were routinely transformed with this vector, the selection of clones was performed as described elsewhere. Recombinant mDD-2 was isolated from bacterial cells by means of affine chromatography on Glutathione-Sepharose (Sigma, USA) with the next proteolysis of GST-mBD-2 fusion protein with thrombin and reverse phase chromatography on Sep-Pack C18 (Waters, USA) carrier similarly to the procedure described earlier for recombinant human beta-defensin-2 [16].

Gene cloning, plasmid constructions and transfection of 3LL cells. The gene coding mature murine β-defensin-2 was cloned from total RNA isolated from murine BALB/c macrophages incubated for 24 h with 10 ng/ml lipopolysaccharides of *P. aeruginosa* and cDNA was amplified by RT-PCR using specific primers (mBD-2-F: 5`-ACCTAAGCT-TCGAACTTGACCACTGCCACACC-3` and mBD-2-R: 5`-GGCGAATTCTCATTTCATGTACTTGCAACAGGG-3). The PCR products were digested with HindIII and EcoRI and cloned into the same sites of expression vector pcDNA3.1+, which contained a selective marker — the neomycin phosphotransferase gene. The constructs were verified by the DNA dideoxy sequencing method and purified using a standard protocol.

To receive a variant for secreted form of mBD-2, leader sequence of Igk gene was recloned from pSec-Tag2A vector (Invitrogene) and placed into pcDNA3.1+ vector before mBD-2 sequence.

The resulting constructs were transfected into 3LL cells using FuGENE 6 Reagent (Roche Molecular Biochemicals) according to the instructions of the manufacturer.

After transfection, the cells were selected in the medium containing 600 μg/mL geneticin G418 (Gibco, Grand Island, NY, USA) for 2 weeks, and stable cell sublines 3LL-pcDNA3 (transfected with blank pcDNA3 vector), 3LL-mBD2 (expressing mBD2) and 3LL-Igk-mBD2 (carrying secreted form of mBD2) were obtained.

RT-PCR analysis of mBD2 expression. Total RNA was isolated from tissue samples by the method of Chromzynski and Sacchi [17]. Concentration of RNA was evaluated at the wave length of 260 nm using Beckman DU-8B spectrophotometer, its purity — by OD relation at 280 nm and 260 nm, its quality — by electrophoresis in 1% agarose gel containing 20% formaldehyde. For detection of mBD-2 RNA expression, semiquantitive RT-PCR analysis was performed with the use of specific primers. The expression level of beta-actine served as the house-keeping gene. The products of RT-PCR were routinely analyzed by electrophoresis in agarose gel. The relative expression level was analyzed with the use of TotalLab Program.

^{3}H-thymidine incorporation. To evaluate cell proliferation rate, radioisotope method was routinely applied as described elsewhere [18]. Shortly, the cells (1 x 10^5 cells/well) were grown on 24-well plate in DMEM for 24 h. Then 0.1 μCi-^{3}H-thymidine per well was added, and the cells were incubated for 16 h. The radioactivity of the cells was measured according standard procedure using scintillation beta counter Beckman 5801 LS.

Colony formation analysis. Analysis of colony forming ability of the cells was carried out by routine technique as described elsewhere [19] in DMEM medium supplemented with 15% FBS and 0.4% methylcellulose. The cells were plated in 6-well plates (1 x 10^3 cells per 1 ml of medium) and incubated in CO_2-incubator for 14–16 days. The colonies were visualized using staining procedure with 0.2% p-iodonitrotetrazolium violet dye and counted.

In vivo study. For *in vivo* research, male C57BL mice weighing 20–25 g bred in the vivarium 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.

3LL cells and transfected cell sublines were transplanted i.m. ((3 x 10^5 cells/100 μl PBS per animal) to C57BL mice mice. Animals were housed in 4 groups (5 mice per group): animals with transplanted wild-type 3LL cells (group 1), 3LL-pcDNA3 cells (group 2), 3LL-mBD2 cells (group 3), and 3LL-Igk-mBD2 cells (group 4). Tumor growth dynamics was monitored each week for 3 weeks by means of calipers. At the day 21 after tumor cell transplantation, the animals were sacrificed by ether narcosis, tumors were removed and weighted, and lung metastases were calculated. By this scheme, 3 independent *in vivo* experiments were performed.

Statistical analysis. The data are reported as the mean ± SD. The statistical significance of differences

between mean values was assessed by the Student's *t*-test. Values $p < 0.05$ were considered statistically significant.

RESULTS

Generation of recombinant mBD-2. For production of recombinant mBD-2, the GST-expression system was chosen. Respectively, recombinant mBD-2 fused with GST was isolated from transformed bacterial cells by means of affine chromatography on Glutathione-agarose with the next proteolysis of GST-mBD-2 fusion protein with thrombin and reverse phase chromatography on Sep-Pack C18 carrier (Fig. 1, *a*) similarly to the procedure described earlier for recombinant human beta-defensin-2. The obtained mBD-2 protein was demonstrated to be active in micromolar concentrations against *P. aeruginosa* (Fig. 1, *b*).

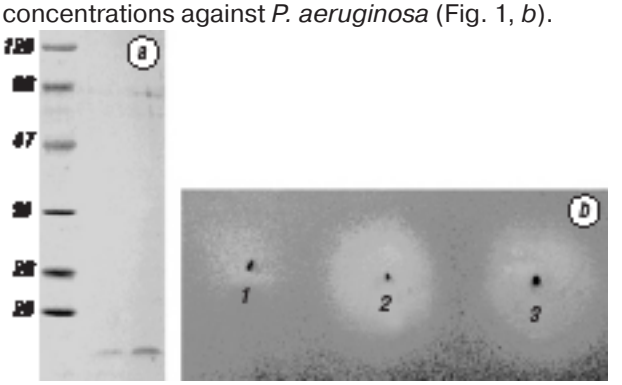

Fig. 1. *a*, Electrophoretic analysis of purity of the recombinant mBD-2 preparation after reverse phase chromatography on Sep-Pack C18. Line 1 — molecular weight markers (Fermentas, Lietuva); lines 2 and 3 — elution with 30% CH3CN and 40% CH3CN, respectively. *b*, Antimicrobial activity of recombinant mBD-2 against *P. aeruginosa*: 1, 2, 3 — 1, 3 and 5 μM mBD-2

So, in this first part of our work, recombinant antimicrobially active mBD-2 was obtained for some special tasks of the further research.

Generation of pcDNA3-Igk-pcDNA3 vectors, transfection of 3LL cells and expression of mBD-2 mRNA in vitro. At the first stage of the research, using standard cloning technique, we have created two vectors at the base of pcDNA3.1+: pcDNA3-mBD-2 and pcDNA3-Igk-mBD-2 that codes mature mBD-2 molecule and its secreted form, respectively. These vectors and blank control pcDNA3.1+ vector were used for the transfection of 3LL cells. After selection on G418 containing medium, the transfected cells were multiplied and analyzed for expression of mBD-2 mRNA.

3LL cells are expressing mBD-2 mRNA at a constant moderate level. Performing such transfection, we supposed that overexpression of mBD-2 mRNA in 3LL cells will be achieved. Surprisingly, finally we have observed quite the opposite effect: in the cells transfected with pcDNA3-mBD-2 and pcDNA3-Igk-mBD-2 (3LL-mBD2 and 3LL-Igk-mBD2 respectively), a notable down-regulation of *mBD-2* gene was registered (Fig. 2), while in the cells transfected with a blank vector this parameter did not differ significantly from that in parental 3LL cells ($p < 0.01$).

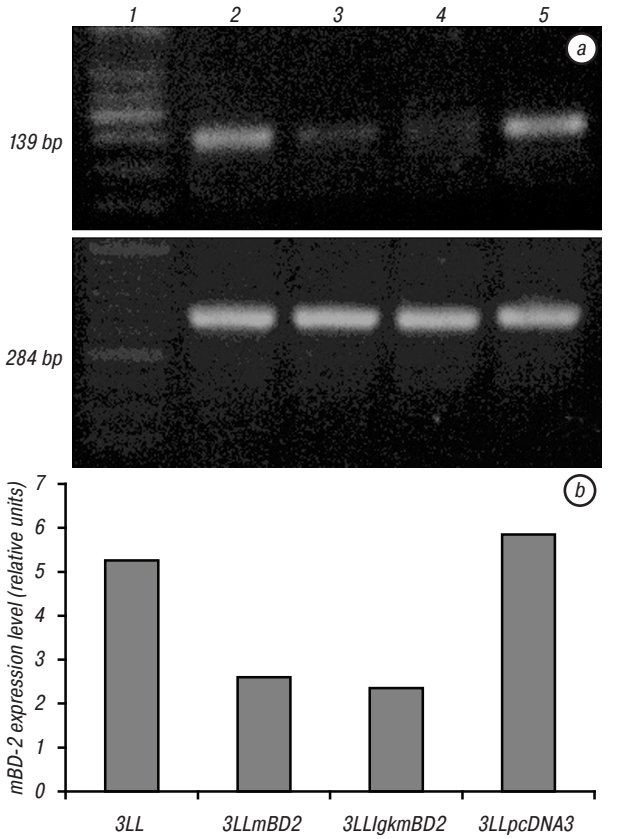

Fig. 2. *a*, RT-PCR analysis of *mBD-2* gene expression in 3LL cells (2), 3LL-mBD2 cells (3), 3LLIgk-mBD2 cells (4), 3LL-pcDNA3 cells (5); line 1 — DNA standard 50 bp ladder (Fermentas, Lietuva) (*a*). Expression of beta-actine as house-keeping gene (*b*). *b,* The data of *mBD-2* gene expression in transfected cells are normalized by beta-actine expression using TotalLab program

To find out whether down-regulation of *mBD-2* gene may be caused by its own protein product, we have performed *in vitro* culturing of wild-type 3LL cells with exogeneously added 0.1 and 1.0 μg/ml recombinant mBD-2 in serum-free medium for 24 h and 48 h. Then the cells were lyzed, total RNA was isolated, and semi-quantative RT-PCR analyzis was carried out. Similarly to the above-mentioned data, in the cells cultivated with recombinant mBD-2 significant decrease of mBD-2 mRNA was detected, and mBD-2 mRNA was nearly undetectable after 48 h incubation of 3LL cells with 1.0 μg/ml recombinant mBD-2 (Fig. 3).

So, on the base of 3LL cells expressing mBD-2 mRNA we have generated the cell sublines 3LL-mBD2 and 3LL-Igk-mBD2 where expression of mBD-2 is strongly down-regulated, possibly, by the feedback mechanism. So, it looked reasonable to check the patterns of 3LL cells with down-regulated *mBD-2* gene compared with parental strain. 3LL cells are widely used in vivo for generation of aggressive experimental murine tumors with high metastatic activity resulting in multiple lung metastases. So, we have checked the proliferative rate and colony forming capacity of sublines 3LL-mBD2 and 3LL-Igk-mBD2 compared to wild type 3LL cells as well as the growth dynamics of tumors that arised from transplanted 3LL cell sublines.

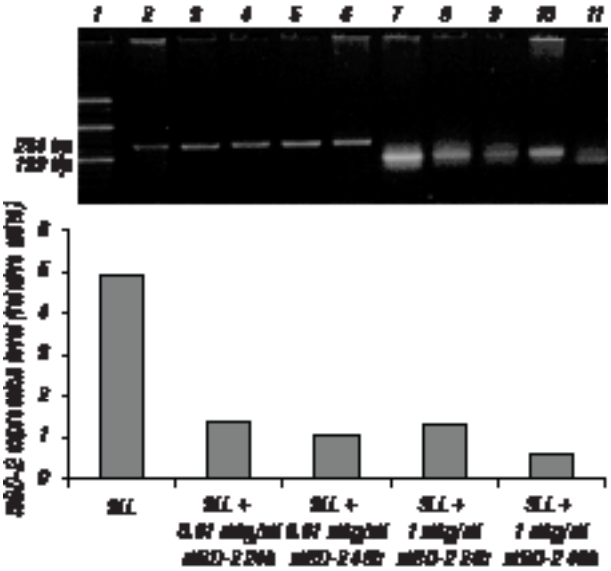

Fig. 3. RT-PCR analysis of *mBD-2* gene expression in 3LL cells incubated in vitro with the recombinant mBD-2. Lines: 1 — DNA standard 50 bp ladder (Fermentas, Lietuva); 2–6 — expression of beta-actine as house-keeping gene; expression of mBD-2 in control 3LL cells (7), 3LL cells induced by 0.01 μg/ml mBD-2 for 24 h (8), 0.01 μg/ml mBD-2 for 48 h (9); 1 μg/ml mBD-2 for 24 h (10), 1 μg/ml mBD-2 for 48 h (11)

Colony formation and proliferation rate of 3LL cells transfected with pCDNA3- pcDNA3 and pCDNA3-Igk-pcDNA3plasmids. Ability to form colonies in a semi-soft medium is an important characteristic of cell culture indicating its tumotigenic potential. The analysis of ability of 3LL, 3LL-pcDNA3, 3LL-mBD2 and 3LL-Igk-mBD2 cells to form colonies in 0.4% methylcellulose has demonstrated that the cells where expression of *mBD-2* gene is down-regulated, formed significantly higher number of colonies than those where expression of mBD-2 mRNA is not affected, and that 3LL-mBD2 and 3LL-Igk-mBD2 cells gave rise to larger colonies than 3LL and 3LL-pcDNA3 did (Table).

Table. Colony forming activity of 3LL cells and 3LL-mBD2 and 3LL-Igk-mBD2 sublines

Cell line	Number of colonies	Colony size
3LL	< 20	< 10 cells
3LL-pcDNA3	< 20	< 10 cells
3LL-mBD2	> 50	> 10 cells
3LL-Igk-mBD2	> 50	> 10 cells

Analysis of ^{3}H-thymidine incorporation in DNA of 3LL, 3LL-pcDNA3, 3LL-mBD2 and 3LL-Igk-mBD2 cells has shown that 3LL-mBD2 and 3LL-Igk-mBD2 are characterized by significantly higher proliferation rate ($p < 0.001$) than the control cells, while this index did not differ significantly between wild-type 3LL cells and the cells transfected with the blank vector ($p > 0.05$) (Fig. 4).

So, our data have indicated an existence of reverse relation between colony-forming activity and proliferation potential of 3LL cells and the level of mBD-2 mRNA expression. The next question to be answered was whether such properties will influence the development and growth dynamics of experimental 3LL tumors that arise from trasfected 3LL cells.

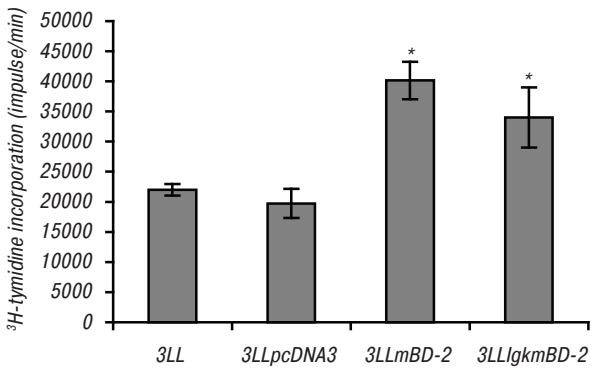

Fig. 4. Analysis of 3H-thymidine incorporation in the DNA of 3LL, 3LL-pcDNA3, 3LL-mBD2 and 3LL-Igk-mBD2 cells. The data (Mean ± SD) of two independent experiments performed in four parallels each are presented
*$p < 0.001$ compared to 3LL.

Influence of mBD-2 expression on the growth dynamics of experimental murine tumors. To answer this question, 3LL, 3LL-pcDNA3, 3LL-mBD2 and 3LL-Igk-mBD2 cells were routinely transplanted (3 x 10^5 cells per animal) in C57 Black mice, and tumor growth dynamics was monitored each week during 21 days period. Our data have shown that the tumors that arised from 3LL-mBD2 and especially from 3LL-Igk-mBD2 cells possessed significantly higher growth rate that was nearly 1.5-fold and 1.7-fold higher than that of 3LL- and 3LL-pcDNA3-derived tumors ($p < 0.05$) (Fig. 5), while 3LL- and 3LL-pcDNA3 tumors did not differ significantly in the growth dynamics ($p > 0.05$). At the day 21 after tumor cell transplantation, the metastatic rate was high in all 4 studied groups, and it was impossible to make correct comparisons between the groups; however, there was observed a tendency for more aggressive metastasis in the case of 3LL-Igk-mBD2-derived tumors (data not shown).

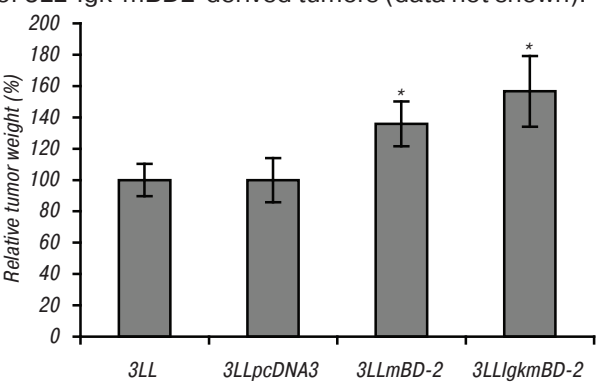

Fig. 5. Relative weight of the tumors derived from transplanted 3LL, 3LL-pcDNA3, 3LL-mBD2 and 3LL-Igk-mBD2 cells at the day 21 after tumor cell transplantation. The data (Mean ± SD) of three independent experiments are presented
*$p < 0.01$ compared to 3LL.

So, these results have demonstrated that down-regulation of *mBD-2* gene expression resulted in acceleration of the development of experimental murine tumors; which, taking into account the above-mentioned data on *in vitro* consequences of mBD-2 down-regulation, may suggest a tumor-suppressing role of murine beta-defensin-2 expression.

DISCUSSION

During the last few years, the defensins became a subject of intensive research due to their recently revealed multiple biological activities that allow to consider them not only as an important component of host antibacterial defense, but as an agents influencing tumorigenesis as well. First of all, *in vitro* studies have shown mitogenic action of defensins, for example, pro-proliferative effects of alpha- and beta-defensins (HNP-1 and hBD-2) at micromolar concentration range toward conjunctival fibroblasts [20] and airway epithelial cells [11]; similar data were reported in our earlier publications where we have shown a dose-dependent mitogenic effect of hBD-2 on human cancer A431 and HeLa cells [13]. However, there are also reports where 0.1–10 μg hBD-2 has no influence of proliferation rate of intestinal epithelial cells, but strongly promotes epithelial cell survival [14].

In the present study, we have introduced an additional copy of *mBD-2* gene in 3LL cells with an aim to receive its hyperexpression to achieve possible suppression of 3LL growth *in vivo*, but received quite the opposite effect and revealed an interesting phenomenon: if expression of *mBD-2* gene was down-regulated, 3LL cancer cells acquire significantly higher proliferative rate ($p < 0.001$). Moreover, such aggressive growth pattern was analogous *in vivo*, when these cells with down-regulated *mBD-2* gene gave rise to experimental tumors. We propose a hypothesis that in fact mBD-2 may play a role of anti-mitogenic molecule that, being expressed at physiologically normal low level, protects cells from excessive proliferation. Some evidence presented in this report point to the existence of some kind of a feedback mechanism: excessive defensin production may result in the down-regulation of its own gene, allowing in turn the cell proliferation. In our earlier studies we did not check expression levels of hBD-2 in the A431 and HeLa cells treated with extracellular defensin; it could not be excluded that in the case of hBD-2 the mechanism of action could be similar. In part, such hypothesis is supported by our research of human embryonal kidney (HEK293) cells transfected with Igk-hBD-2-containing vector (not published data): if wild-type cells do not express hBD-2 and possess high proliferative rate and high colony forming activity, transfected cells are expressing picomolar concentrations of hBD-2 and largely lost colony formation ability along with decreased proliferation rate. Of course, such hypothesis requires further more intense studies for its support. At the same time, these unexpected data received in *in vitro* and *in vivo* models are in agreement with an opinion about possible tumor-suppression functions of defensins [21] delineated from a number of recent studies including those carried on human prostate and renal tumors [7]. Fundamental studies of defensin signaling machinery will strongly facilitate our knowledge on exact functional role of these antimicrobials in cancer cells.

ACKNOWLEDGEMENTS

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СНИЖЕНИЕ УРОВНЯ ЭКСПРЕССИИ БЕТА-ДЕФЕНСИНА-2 МЫШИ В КЛЕТКАХ КАРЦИНОМЫ ЛЕГКИХ ЛЬЮИС ПРИВОДИТ К УСКОРЕНИЮ РОСТА ОПУХОЛЕВЫХ КЛЕТОК *IN VITRO* И *IN VIVO*

***Цель*: настоящая работа посвящена анализу противоопухолевых свойств бета-дефенсина-2 мыши (mBD-2) *in vitro* and *in vivo*. *Материалы и методы*: на основе pcDNA3.1+ вектора были созданы 2 плазмидных конструкта, кодирующих зрелую форму mBD-2, содержащие или несодержащие сигнальную последовательность Igk (pcDNA3mBD-2 и pcDNA3Igk-mBD-2 соответственно). Путем трансфекции клеток 3LL полученными векторами, а также контрольным вектором pcDNA3.1+ были получены клеточные линии (3LL-mBD-2, 3LL-Igk-mBD-2 и 3LL-pcDNA3), для которых были проведены исследования их пролиферативной активности, определенной по уровню включения ^{3}H-тимидина в ДНК, и способности к колониеобразованию в среде, содержащей метилцеллюлозу. Экспрессию гена *mBD-2* исследовали с помощью полуколичественного ОТ-ПЦР-анализа. Трансфецированные клетки были имплантированы мышам линии C57BL, после чего была проанализирована динамика роста опухоли. *Результаты*: установлено, что в трансфектных клеточных линиях 3LL-mBD-2 и 3LL-Igk-mBD-2 уровень экспрессии mBD-2 снижен по сравнению с контрольными. Эти клетки характеризовались достоверным повышением уровня пролиферации ($p < 0,001$) и способности к колониеобразованию. Клетки сублиний 3LL-mBD-2 и 3LL-Igk-mBD-2, трансплантированные мышам линии C57BL, вызывали развитие более агрессивных опухолей, обладающих значительно более высокой скоростью роста ($p < 0,01$), чем таковые, вызванные перевивкой клеток 3LL. *Выводы*: полученные результаты свидетельствуют о возможной роли mBD-2 как опухолевого супрессора.**

***Ключевые слова*: бета-дефенсин-2 мыши, карцинома легких Льюис (3LL), экспрессия, пролиферация, колониеобразование.**

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