INVOLVEMENT OF HUMAN BETA-DEFENSIN-2 IN REGULATION OF MALIGNANT POTENTIAL OF CULTURED HUMAN MELANOMA CELLS

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Background and Aim: Human beta-defensin-2 (hBD-2) is an antimicrobial cationic peptide capable to control human carcinoma cell growth via cell cycle regulation. The present study was aimed on determination of hBD-2 influence on the growth patterns and malignant potential of cultured human melanoma cells. ***Methods***: The study was performed on cultured human melanoma cells of mel Z and mel Is lines treated with recombinant hBD-2 (rec-hBD-2); cell viability, proliferation, cell cycle distribution, and anchorage-independent growth were analyzed using MTT test, direct cell counting, flow cytometry, and colony forming assay respectively. Expression and/or phosphorylation levels of proteins involved in cell cycle control were evaluated by Western blotting. ***Results***: The treatment of mel Z and mel Is cells with rec-hBD-2 in a concentration range of 100−1000 nM resulted in a concentration-dependent suppression of cell proliferation, viability, and colony forming activity. It has been shown that rec-hBD-2 exerts its growth suppression effects via significant downregulation of B-Raf expression, activation of pRB and upregulation of p21^{WAF1} expression, downregulation of cyclin D1 and cyclin E resulting in cell cycle arrest at G1/S checkpoint. ***Conclusion***: According to obtained results, hBD-2 exerts its growth suppression effect toward human melanoma cells via downregulation of B-Raf, cyclin D1 and cyclin E expression, upregulation of p21^{WAF1} expression and activation of pRB.
Key Words: human beta-defensin-2, melanoma, proliferation, viability, cell cycle, B-Raf, anchorage-independent growth.

Human melanoma is the most lethal form of skin cancer with constantly rising incidence worldwide. While early stage melanoma is curable, melanoma patients with advanced disease have poor prognosis because this type of cancer is resistant to chemotherapy [1]. That's why there is a need for the development of new strategies for melanoma treatment.

Cationic antimicrobial peptides (CAPs) of eukaryotic origin are among many agents with potential anti-melanoma activity. These peptide antibiotics are produced by many living species including mammals. CAPs are active against different bacterial pathogens, viruses, and fungi. Therapeutic potential of peptide antibiotics as anti-infective agents is considered promising despite the difficulties related to their pharmacokinetics, toxicity and immunogenicity [2]. Moreover, some of CAPs exert cytotoxic activity toward cancer cells. This ability of CAPs is thought to be related to the biophysical properties of their molecules, in particular high cationic charge and amphipathicity allowing interaction of CAP molecules with negatively charged cancer cell membranes and cell lysis [3]. The knowledge on cytotoxic activity of particular CAPs against cancer cells *in vitro* and *in vivo* has been gained. Moreover, a number of these peptides have been studied in experimental models as anticancer agents [4]. In particular, some antimicrobial peptides, like cathelicidin-BF from the venom of the snake *Bungarus fasciatus* [5], crotamine from the venom of *Crotalus durissus terrificus* rattlesnake [6], or gomesin from hemocytes of the spider *Acanthoscurria gomesiana* [7] have been demonstrated to be effective for experimental B16 melanoma treatment *in vivo*.

In humans, CAPs are represented by the host defense peptides of defensin family (human α- and β-defensins, hBDs) and cathelicidin LL-37, important components of innate immunity protecting organism from pathogens along with many other biologically active molecules [8, 9]. It has been assumed that hBDs and LL-37 play a critical role in antimicrobial protection of human skin and in wound healing processes, and dysregulation of hBDs expression (in particular, hBD-2) is implicated in pathogenesis of many skin pathologies, in particular, psoriasis and atopic dermatitis [10–12]. At the same time, despite the steady progress in understanding hBDs role in inflammatory skin disorders, little is known about possible implication of these peptides in the development of skin cancer.

It has been shown recently that in cutaneous squamous cell carcinoma (SCC) hBD-1 is downregulated, while hBD-2 and hBD-3 are upregulated compared to healthy skin. So, the authors supposed that in SCC hBD-1 may play a role of tumor suppressor, while hBD-2 and hBD-3 may promote skin cancer development [13]. However, hypothetically, the resulting effect of hBDs expression could depend on many factors, including their local concentrations in particular tumor mi-

croenvironment as far as these antimicrobials are shown to exert different effects in a concentration-dependent manner [14–17]. For example, hBD-2 and hBD-4 in low nanomolar concentration range stimulate carcinoma cell proliferation, but at higher doses are capable to block cell cycle and drastically suppress cancer cell growth *in vitro* [15, 17]. In high concentrations some human defensins exert potent cytotoxic activities against cancer cells *in vitro* and *in vivo*, similarly to CAPs of other origin [18, 19].

Up-to-date, the effects of hBDs in physiological (nanomolar) concentration range on melanocytes and melanoma cells remains poorly studied. Melanoma arise from melanocytes, pigment dendritic cells located in epidermis and hair follicles. Theoretically, their behavior could be regulated through paracrine way by defensins produced by neighboring keratinocytes. Indeed, it has been established that one of the inducible β-defensins, namely hBD-3, expression of which could be induced in keratinocytes by UVR [20], in low nanomolar concentrations exerts pigmentary effects and is a high-affinity ligand of melanocortin-1 receptor (MC1R). It is well recognized that MC1R is a receptor, which play a central role in pigmentation and in melanoma susceptibility in humans. It is supposed that hBD-3 may play an important role in melanocyte biology, response of human skin to UVR and skin cancer susceptibility [21, 22]. The effects of other hBDs on melanoma cells remain unknown.

In the present study we addressed a question whether exogenous recombinant hBD-2 (rec-hBD-2) could regulate the growth patterns and malignancy potential of cultured human melanoma cells. We have revealed that proliferation and viability of human melanoma cells are regulated by hBD-2 in a concentration-dependent manner. In particular, rec-hBD-2 in high nanomolar range suppresses viability and proliferation of melanoma cells via cell cycle arrest in G1/S checkpoint and markedly reduces their colony forming activity. Significant downregulation of B-Raf, cyclin E and cyclin D1 expression along with dephosphorylation of pRB in human melanoma cells treated with hBD-2 have been demonstrated. These data may be useful for further development of new approaches for melanoma treatment using CAPs.

MATERIALS AND METHODS

Cell lines and recombinant hBD-2 peptide. The study was carried out on 2 human melanoma cell lines — mel Z and mel Is, which were kindly provided by prof. A.Y. Baryshnikov (N.N. Blokhin Russian Cancer Research Center RAMS, Moscow, Russian Federation). Mel Z and mel Is cell lines were established from surgically resected metastatic lesions of patients with malignant disseminated melanoma by Dr. O.S. Burova (N.N. Blokhin Russian Cancer Research Center RAMS, Moscow, Russian Federation) [23].

Cell lines were cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate in humidified 5% CO_2 atmosphere at 37 °C.

To study the effect of exogenous defensin on cell growth, we used purified recombinant hBD-2 [24].

In brief, *E. coli BL21(DE3)* cells transformed with GST-hBD-2-recombinant plasmid were induced with 1 mM IPTG for 6 h, pelleted by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.6; 250 mM NaCl; 1% Triton X-100 and cocktail of protease and phosphatase inhibitors), and disrupted using ultrasound disintegrator (UD-11 Automatic, Poland). Then cell lysate was applied to affinity chromatography on glutathione-agarose column (GE Healthcare, Sweden) with following cleavage of the defensin from fusion protein by thrombin digestion. hBD-2 peptide was further purified by reverse phase chromatography on Sep-Pack C18 cartridge (Waters, USA), vacuum-dried, and re-dissolved in acidified water. Protein concentration was determined by UV absorbance at 280 nm using spectrophotometer Nanodrop-1000 (USA).

Direct cell counting. To study the effect of rec-hBD-2 on cell proliferation, mel Z and mel Is cells were routinely cultured in 24-well plates ($5 \cdot 10^4$ cells per well) to nearly 50% confluence, then culture medium was replaced with fresh DMEM supplemented with 2.5% FBS. Rec-hBD-2 was added into the medium in concentrations of 100, 500, or 1000 nM, followed by culturing for 48 h. After the treatment, cells were washed with PBS, detached with trypsin, and counted in hemocytometer. The percentage of dead cells was analyzed using trypan blue staining.

MTT assay. To evaluate the effect of rec-hBD-2 on cell viability, MTT-test has been applied [25]. Mel Z and mel Is cells were seeded into 96-well plates ($7 \cdot 10^3$ cells per well) and incubated with rec-hBD-2 at the concentration range from 1 pM to 2 µM in DMEM supplemented with 2.5% FBS for 48 h. Then cells were routinely treated with MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) according to standard protocol, and colorimetric reaction was evaluated with the use of ELISA reader (Awareness Technology Inc, USA) at λ = 545.

Flow cytometry analysis. Mel Z and mel Is cells were cultured in 10 cm Petri dishes in DMEM supplemented with 10% FBS to 50% confluence, then the medium was replaced by the fresh DMEM supplemented with 2.5% FBS, and cells were treated with 1, 10, 100, 500, or 1000 nM rec-hBD-2 for 48 h. After the treatment, the attached cells were washed with PBS, harvested by trypsinization, pelleted at 4 °C (500 g) for 5 min, washed twice in PBS, and resuspended in 1 ml of hypotonic cell lysis buffer (0.1% sodium citrate, 0.1% Triton X-100, 5 µg/ml propidium iodide (Sigma, USA)). Cells were incubated at RT for 30 min in the dark, and cell cycle distribution was analyzed using cytometer Becton Dickinson FACS Calibur. The data were analyzed with the use of CellQuest software package and ModFit LT2.0 program (BDIS, USA) for Mac computers.

Western blot analysis. To analyze the level of expression and/or phosphorylation of some components of signaling pathways involved in cell cycle regulation, melanoma cells were cultured in 6-well plates and treated with 1000 nM rec-hBD-2 for 48 h as described above, washed with PBS and lyzed in RIPA buffer

with protease and phosphatase inhibitors. Proteins were separated in 9–22% gradient SDS-PAGE and transferred to nitrocellulose membrane Hybond-ECL, RPN3032D (Amersham Biosciences, USA). Nonspecific binding sites were blocked with 1X PBS-T, 5% milk solution for 3 h. Then the blots were incubated sequentially with primary Abs, and then with secondary polyclonal HRP-conjugated anti-rabbit IgG or anti-mouse IgG Abs (DakoCytomation, Denmark). The ECL Western blotting detection system (Amersham Pharmacia Biotech) was used to reveal immunoreactivity. For Western blotting, we have used antibodies against pRB, cyclin D1, phospho-pRB (S780), and CDK4 from Cell Signaling Tech (USA), p53 (IEPOR, Ukraine), p21[WAF1] (Oncogene, USA), cyclin E, B-Raf (Santa-Cruz, USA), and beta-actin (Sigma, USA). All antibodies were used at the working dilutions according to manufacturer instructions.

Colony forming assay and melanin content analysis. Substrate independent growth was studied by routine procedure [26]. The cells were seeded in 3 cm Petri dishes ($5 \cdot 10^3$ cells) in the medium containing 0.5% methylcellulose, DMEM, 15% FBS with or without addition of 100, 500, or 1000 nM rec-hBD-2. After 2-weeks culturing in 5% CO_2 at 37 °C, the developed colonies were stained with 0.01% neutral red in PBS for 30 min, and examined by light microscopy. TotalLab 2.0 program (UK) has been used for data evaluation.

Melanin content in cultured human melanoma cells was analyzed using standard Fontana — Masson method [27].

Statistical analysis. The data are reported as the mean ± SD. Data on direct cell counting, MTT, flow cytometry analysis and colony forming assay were analyzed by Student's *t*-test to assess the statistical significance of the difference between the groups. A statistically significant difference was considered to be present at $p < 0.05$.

RESULTS

The study has been performed *in vitro* using two human melanoma cell lines — mel Z cells originating from moderately differentiated melanoma, and mel Is originating from low differentiated melanoma [23].

The analysis of rec-hBD-2 effect on proliferation and viability of cultured human melanoma cells. To study the effect of rec-hBD-2 on growth rates of cultured melanoma cell lines, direct cell counting technique has been used. Mel Z and mel Is cells were treated with rec-hBD-2 that was added into cell culture medium at different concentrations (100 nM, 500 nM, 1 μM) for 48 h. Rec-hBD-2 caused significant concentration-dependent suppression of mel Z proliferation ($p < 0.05$), while in mel Is cells growth suppression has been observed only in the case of treatment with 500 nM and 1 μM rec-hBD-2 ($p < 0.05$) (Fig. 1). Effect of the defensin on melanoma cell proliferation was hBD-2-specific because preincubation of rec-hBD-2 with anti-hBD-2 Abs (Santa-Cruz, USA) prior to its addition into cell culture medium completely abolished the above mentioned effects. According

to the data of trypan blue staining, the percentage of dead cells in all samples didn't exceed 3.5–4% for all tested rec-hBD-2 concentrations.
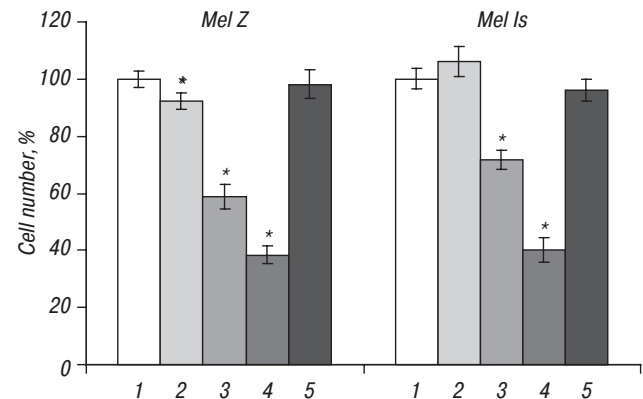

Fig. 1. A concentration-dependent effect of rec-hBD-2 on the number of viable cultured human melanoma cells of mel Z and mel Is lines. Mel Z and mel Is cells were treated with rec-hBD-2 in concentrations of 100 nM (columns 2), 500 nM (column 3), 1000 nM (column 4); column 1 — control cells; column 5 — the cells were treated with 500 nM rec-hBD-2 after its 30 min preincubation with anti-hBD-2 antibodies (Santa-Cruz, USA). The number of attached cells was evaluated by direct cell counting. The data of three independent experiments are presented as the mean ± SD. *The difference is significant as compared to appropriate control ($p < 0.05$)

Next, we have analyzed the effect of rec-hBD-2 on viability of cultured mel Z and mel Is cells using MTT assay. It has been shown that at low concentrations (1 pM — 1 nM) rec-hBD-2 didn't affect significantly viability of both cell lines, while treatment of the cells with higher concentrations of rec-hBD-2 (10 nM — 1000 nM) resulted in significant dose-dependent decrease of cell viability ($p < 0.05$) (Fig. 2).
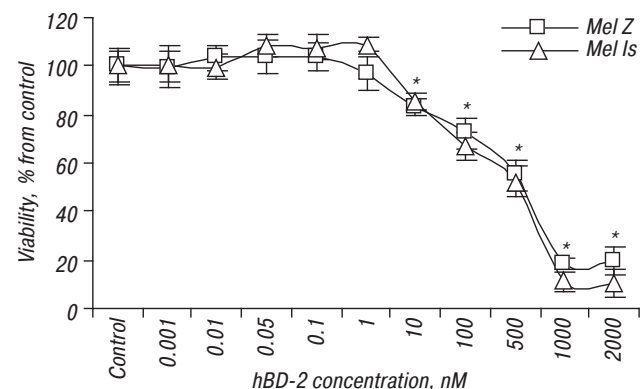

Fig. 2. A concentration-dependent effect of rec-hBD-2 on viability of mel Z and mel Is cells. Cell viability was evaluated by MTT analysis. The data of three independent experiments are presented as the mean ± SD. *The difference is significant as compared to appropriate control ($p < 0.05$)

So, the data of direct cell counting and MTT demonstrated that low-differentiated melanoma mel Is cells are less sensitive to growth suppressing action of rec-hBD-2 than moderately differentiated mel Z cells. Another important observation is an absence of apoptotic effects of the defensin toward cultured melanoma cells: the percentage of dead cells slightly differed between the samples treated with different hBD-2 concentrations. This fact was further confirmed by the data of flow cytometry (see below) and analysis of PARP expression in hBD-2 treated melanoma cells (data not shown).

Rec-hBD-2 affects cell cycle distribution and signaling pathways involved in cell cycle regulation in vitro. Flow cytometry analysis of cell cycle distribution of mel Z cells treated with 10–1000 nM rec-hBD-2 for 48 h demonstrated a concentration-dependent cell growth arrest at G1/S checkpoint ($p < 0.05$) (Fig. 3). Similar results were registered for mel Is cells treated with rec-hBD-2 (data not shown). Treatment of melanoma cells with 10–1000 nM of hBD-2 did not affect the percentage of apoptotic cells (see Fig. 3).
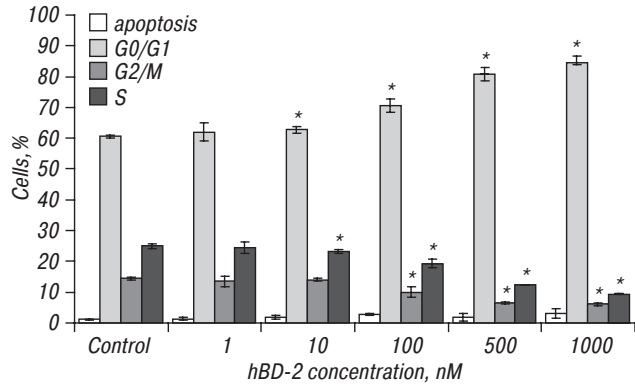

Fig. 3. Flow cytometry analysis of cell cycle distribution of mel Z cells treated with rec-hBD-2. Mel Z cells were treated with 1, 10, 100, 500, or 1000 nM rec-hBD-2 for 48 h. The data of two independent experiments are presented as the mean ± SD. *The difference is significant compared to appropriate control ($p < 0.05$)

To explore the effect of rec-hBD-2 on the proteins involved in cell cycle regulation and cell growth control, the expression levels of cyclin D1, cyclin E, retinoblastoma protein (pRB), CDK4, p21^WAF1^, p53, B-Raf and phosphorylation of pRB in mel Z and mel Is cells treated for 48 h with 1000 nM rec-hBD-2 in comparison to control (untreated) cells were studied using Western blotting. Treatment with rec-hBD-2 resulted in slight downregulation of pRB expression and its complete dephosphorylation. Moreover, we revealed suppression of cyclin D1 expression, significant downregulation of cyclin E, CDK4 and B-Raf expression levels, and significant upregulation of p21^WAF1^ in hBD-2-treated melanoma cells. At the same time p53 expression level remained unaffected (Fig. 4).

Effect of rec-hBD-2 on colony-forming activity of human melanoma cells. We have performed colony forming assay to evaluate an effect of rec-hBD-2 on anchorage-independent growth ability of human melanoma cells. It was shown that treatment with 100 nM of rec-hBD-2 resulted in insignificant decrease of colony numbers compared to control cells, addition of 500 nM of rec-hBD-2 into cell incubation medium significantly inhibited ($p < 0.05$) ability of mel Is cells to form colonies in semi-soft medium (Fig. 5), while in the presence of 1 μM of the defensin no visible colonies were developed. Also, rec-hBD-2 caused a concentration-dependent effect on anchorage-independent growth of mel Z cells (data not shown).
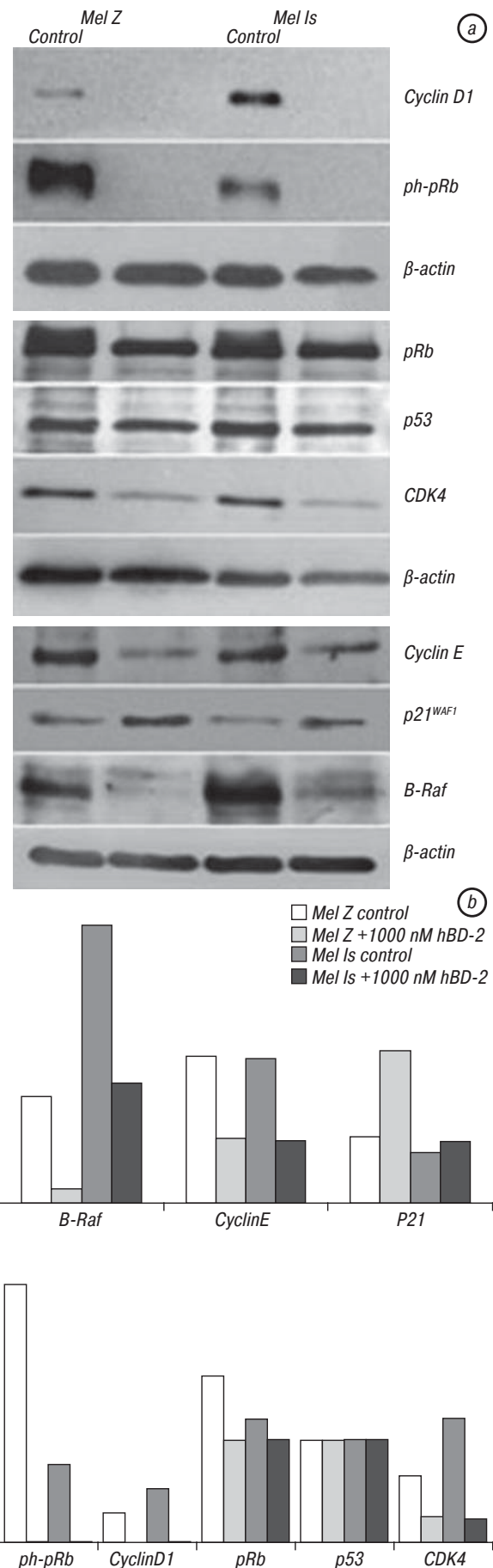

Fig. 4. Western blot analysis of expression levels of cyclin D1, cyclin E, pRB, CDK4, p53, B-Raf, and p21^WAF1^, and phosphorylation of pRB (ph-pRB) in mel Z and mel Is cells treated with 1000 nM rec-hBD-2 for 48 h in comparison to control (untreated) cells (*a*). Graphic representations of Western blotting data calculated with the use of TotalLab program (*b*)
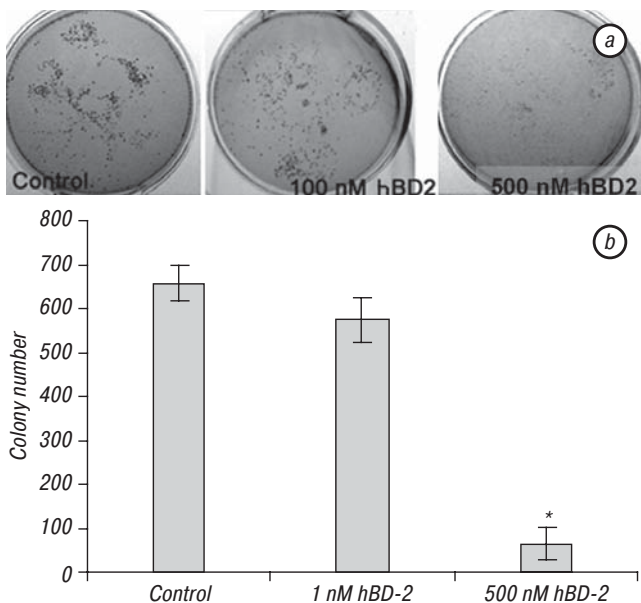
Fig. 5. Rec-hBD-2 significantly inhibits colony-forming activity of mel Is cells. The graph represents the numbers of colonies calculated with the use of TotalLab program

Melanin content in cultured human melanoma cells of mel Z and mel Is cells treated with 1 nM, 100 nM or 500 nM rec-hBD-2 for 72 h was analyzed. We did not found alterations in melanin content in mel Z and mel Is cells treated with the defensin compared to control cells (data not shown). Thus, hBD-2 seems to be not involved in melanin synthesis regulation in cultured human melanoma cells.

DISCUSSION

In the present study we have shown that recombinant hBD-2, cationic host defense peptide, affects proliferation, viability, and cell cycle distribution of human melanoma cells *in vitro*. The effects of rec-hBD-2 on melanoma cell proliferation and viability were found to be similar to those in human A431 and A549 carcinoma cells [15]. Indeed, treatment of mel Z and mel Is cells with rec-hBD-2 at concentrations higher than 100 nM resulted in significant inhibition of cell proliferation and viability evidently realized via cell cycle arrest at G1/S checkpoint.

The mechanism of cell division suppression by the defensin could be possibly based on inhibition of B-Raf expression and inactivation of signaling pathways controlled by this kinase. It is known that decreased expression of B-Raf affects activity of downstream kinases such as MEK1 and ERK, and induction of transcription factors c-Fos, c-Jun and important mediator of growth factors effects — c-Myc, which in turn controls expression of cyclin D1 and CDK4 [28]. It's necessary to note that treatment of melanoma cells with rec-hBD-2 resulted in complete down-regulation of cyclin D1 expression (see Fig. 4). In contrary to cyclins E and B, the quantities of which after termination of their functioning during cell cycle progression decrease to minimal values, cyclin D1 expression remains at relatively high level — approximately equal to its half-maximal value in G1 phase [29].

According to our data (see Fig. 4), the possible mechanism of cell cycle arrest in G1/S checkpoint in melanoma cells treated with rec-hBD-2 could be based on activation (dephosphorylation) of pRB, down-regulation of cyclins D1 and E, and increased quantity of CDK inhibitor — $p21^{WAF1}$. Up-regulation of $p21^{WAF1}$ is difficult to explain because expression of p53 in the melanoma cells treated with rec-hBD-2 did not alter significantly. It can not be excluded that an increase of $p21^{WAF1}$ expression is realized via alternative, p53-independent mechanisms [30, 31].

The mechanism of growth-suppressing activity of rec-hBD-2 toward human melanoma cells *in vitro* was found to be similar to that in human A431 and 549 carcinoma cells — via cell cycle arrest at G1/S checkpoint [15]. Therefore, this activity of the defensin is not cell-type specific.

In current study we found a significant influence of rec-hBD-2 on expression of B-Raf — an important serine/threonine kinase involved in regulation of cell growth. Mutations in *BRAF* gene resulting in constitutive activation of the kinase are often observed in many human cancers, including melanoma [32]. Novel drugs, specific B-Raf inhibitors, have been developed; some of them (dabrafenib, vemurafenib) have been evaluated in clinical trials for melanoma treatment and the results of these trials are considered promising [33]. Our finding, which demonstrated a potent down-regulation of B-Raf expression in human melanoma cells *in vitro* caused by innate defense molecule hBD-2 can be potentially useful in the development of new agents for combined treatment of melanoma.

Also, our data have shown that rec-hBD-2 is capable to suppress an important hallmark of malignant cells — their ability for anchorage-independent growth. Indeed, in the presence of 500 nM of rec-hBD-2 the number of colonies formed by mel Z cells was nearly 10 fold lower that in the control ($p < 0.05$) (see Fig. 5). Similar ability to inhibit malignant potential of human cancer cells *in vitro* has been described recently for human beta-defensin-4 — another inducible peptide antibiotic expressed by epithelial cells [17].

The results of our study raise new questions: do human melanoma cells express β-defensins, and could their behavior and growth patterns be affected by paracrine way through hBDs expressed by neighboring skin keratinocytes? Unfortunately, little is known about expression of hBDs in normal melanocytes and malignant melanoma cells. At the same time, experimental studies have provided evidence on responsiveness of melanocytes to the action of β-defensins. In particular, it has been demonstrated that some human β-defensins (hBD-1 and hBD-3) in low nanomolar concentrations are high-affinity ligands for MC1R in primary human melanocytes, and it has been supposed that hBD-3 may activate MAPK and cAMP signaling pathways via MC1R thus regulating melanocyte responses [22, 34]. In a recent Spanish case-control study [35] it has been shown that hBD-1 (but not hBD-3) could be involved in malignant melanoma

susceptibility as far as polymorphisms in hBD-1 gene may be associated with increased risk of melanoma. According to the data of our research, treatment with rec-hBD-2 had no influence on melanin production levels in mel Z and mel Is cells. At the same time, hBD-2 significantly affects melanoma cell growth patterns and malignancy potential *in vitro* and hypothetically could be capable to exert such effects *in vivo*. It will be reasonable to study possible associations between hBD-2 expression patterns and/or genetic polymorphisms of its gene in malignant melanoma and the risk of the development of this type of cancer.

In conclusion, our data have shown that exogenous hBD-2 can regulate the growth patterns of human melanoma cells in a concentration-dependent manner. In particular, in medium-to-high nanomolar concentration range this peptide is capable to suppress viability and proliferation of melanoma cells via cell cycle arrest in G1/S checkpoint and to reduce significantly their colony forming activity. Importantly, growth-suppressing activity of hBD-2 is mediated by significant down-regulation of B-Raf, cyclin E and cyclin D1 expression along with dephosphorylation of pRB in human melanoma cells. Together, these results provide new evidence on important role of hBD-2 in regulation of malignant potential of human melanoma cells.

COMPETING INTERESTS

ACKNOWLEDGEMENTS

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