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BIOLOGIC ACTIVITIES OF RECOMBINANT HUMAN-BETA-DEFENSIN-4 TOWARD CULTURED HUMAN CANCER CELLS

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***Aim*: The aim of the study was *in vitro* analysis of biological activity of recombinant human beta-defensin-4 (rec-hBD-4). *Methods*: hBD-4 cDNA was cloned into pGEX-2T vector, and recombinant plasmid was transformed into *E. coli* BL21(DE3) cells. To purify soluble fusion GST-hBD-4 protein, affinity chromatography was applied. Rec-hBD-4 was cleaved from the fusion protein with thrombin, and purified by reverse phase chromatography on Sep-Pack C18. Effects of rec-hBD-4 on proliferation, viability, cell cycle distribution, substrate-independent growth, and mobility of cultured human cancer cells of A431, A549, and TPC-1 lines were analyzed by direct cell counting technique, MTT assay, flow cytofluorometry, colony forming assay in semi-soft medium, and wound healing assay. *Results*: Rec-hBD-4 was expressed in bacterial cells as GST-hBD-4 fusion protein, and purified by routine 3-step procedure (affine chromatography on glutathione-agarose, cleavage of fusion protein by thrombin, and reverse phase chromatography). Analysis of *in vitro* activity of rec-hBD-4 toward three human cancer cell lines has demonstrated that the defensin is capable to affect cell behaviour in concentration-dependent manner. In 1–100 nM concentrations rec-hBD-4 significantly stimulates cancer cell proliferation and viability, and promotes cell cycle progression through G2/M checkpoint, greatly enhances colony-forming activity and mobility of the cells. Treatment of the cells with 500 nM of rec-hBD-4 resulted in opposite effects: significant suppression of cell proliferation and viability, blockage of cell cycle in G1/S checkpoint, significant inhibition of cell migration and colony forming activity. *Conclusion*: Recombinant human beta-defensin-4 is biologically active peptide capable to cause oppositely directed effects toward biologic features of cancer cells *in vitro* dependent on its concentration.**

***Key Words*: human beta-defensin-4, cancer cell, proliferation, migration, colony forming activity.**

Human beta-defensins (hBDs), cationic antimicrobial peptides and important components of innate immunity system in humans, are mainly expressed in epithelial cells of different origin and provide first-line defense of epithelial surfaces from microbial challenge. According to modern knowledge, the family of hBDs includes at least 30 members most of which are indentified by computational analysis and functions of which remain unknown [1, 2].

Up to date, the most studied members of beta-defensin subfamily are hBD-1 and hBD-2, and to the lesser extent — hBD-3 and hBD-4. hBD-1 was originally isolated from hemofiltrate in 1995 [3], and later was found in urogenital and respiratory tracts [4]; this defensin is supposed to be constitutively expressed. Three other hBDs are peptides with inducible character of expression. hBD-2 was isolated from psoriatic skin lesions [5] and later from many epithelial tissue types; its expression is shown to be regulated by different bacteria and inflammatory factors due to the presence of binding site for inflammatory mediator NF-κB in the promoter region of *hBD-2* gene [6]. hBD-3 and hBD-4 have been firstly discovered by screening genomic sequences of the Human Genome Project [7, 8]; hBD-3 expression is induced upon stimulation with interferon-γ via STAT binding site in promoter region of hBD-3 gene [7]. Also, in 2001 hBD-3 was isolated from psoriatic lesions, cloned from keratinocytes and expressed as His-Tag-fusion protein in *E. coli* [9]. *hBD-4* gene promoter contains no NF-kB or STAT binding sites, and its expression is positively regulated with PMA and bacteria and is thought to be mediated by protein kinase C [8].

All these beta-defensins demonstrate potent antimicrobial activity against a variety of bacterial pathogens, that's why direct microbial killing is supposed to be their main function. However, experimental data suggest that defensins are multifunctional molecules with a wide spectrum of important biological activities and could be involved not only in local immediate antimicrobial response but also in chemotaxis, modulation of inflammatory response, angiogenesis, and wound healing [10, 11]. The last process is of special interest. It is shown in a number of studies that expression of inducible hBDs is up-regulated in skin lesions after injury and these peptides positively regulate keratinocyte proliferation and migration thus promoting wound healing [12, 13]. In a number of studies there have been documented abilities of hBDs to influence many vital cell processes — cell proliferation, viability, differentiation, and apoptosis, and it has been shown that such effects of hBDs are concentration dependent and could be exerted against many cell types, not only keratinocytes [14–17]. In a similar manner hBDs may

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Abbreviations used: GST – glutathione-S-transferase; hBDs – human beta-defensins; hBD-4 – human beta-defensin-4; rec-hBD-4 – recombinant hBD-4.

affect growth patterns of tumor cells and may play a role in promotion or suppression of human cancer cell growth.

In recent years, new insights on possible involvement of hBDs in tumorigenesis have been gained [17–27]. hBD-1 is considered a tumor suppressor: its expression is absent in prostate cancer and renal clear cell carcinoma; down-regulation of hBD-1 expression contributes to cancer cell survival while its induction results in tumor cell death [17, 18]. The role of hBD-2 in cancer cell biology is not so evident because in different tumor types expression patterns of this defensin could differ significantly. In salivary gland tumors hBD-2 expression is down-regulated [19]; the authors suppose that in salivary gland hBD-2 may play antioncogenic functions [19]. According to our data [20, 21], in lung adenocarcinoma expression level of hBD-2 correlates with tumor differentiation grade, while *in vitro* this defensin causes significant suppression of lung cancer cell growth via cell cycle regulation. In oral squamous cell carcinoma hBD-2 has been shown to be up-regulated and is supposed to play a pro-oncogenic role [22]. So, functional meaning of hBD-2 expression in cancer cells seems to depend on histologic type of tumor and on its microenvironment. This is true also for hBD-3 which is considered as pro-oncogenic molecule in some tumors (head and neck cancer, oral carcinoma [23, 24]) or tumor suppressor in salivary gland tumors [19]. Up to date, the role of hBD-4 in tumor cell biology has not been analyzed.

In this work we report successful production of biologically active recombinant hBD-4 (rec-hBD-4) expressed in prokaryotic cells. Analysis of biologic activity of this defensin against cultured human cancer cells has shown that hBD-4 is capable to affect *in vitro* human tumor cell proliferation and viability, migration potential and substrate-independent growth in a concentration-dependent manner characteristic to other representatives of beta-defensin family.

MATERIALS AND METHODS

Cell lines. Human epidermoid carcinoma A431 cells and human lung adenocarcinoma A549 cells were obtained from the Bank of Cell Lines from Human and Animal Tissues, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). Human papillary thyroid carcinoma cell line TPC-1 was kindly provided by Dr. V.M. Pushkarev (V.P. Komissarenko Institute of Endocrinology and Metabolism, AMS of Ukraine, Kyiv, Ukraine). The cells were cultured *in vitro* in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate in 5% CO_2 atmosphere at 37 °C.

The cloning of hBD-4 and its prokaryotic expression. To clone the gene coding for mature hBD-4 sequence, total RNA was isolated from human lung squamous cell carcinoma sample No 5 [27], and hBD-4 cDNA was obtained by reverse transcription using a pair of specific primers: hBD-4-F: 5`-GTGTTGGATCCGAATTTGAATTGGACAGAAT-3`; hBD-4-R: 5`-TCTTGGAATTCTCAGGGTTTTGTACGATTCAGTA-3. Primer design was based on analysis of NM_080389.2 sequence from GenBank database.

HBD-4 cDNA was cloned into pGEX-2T vector (GE Healthcare, Sweden). Restriction of plasmids, ligation of fragments, bacterial cell transformation were performed by standard protocols.

Production and purification of rec-hBD-4. Rec-hBD-2 was purified by three-step procedure similarly to rec-hBD-2 [28]. Shortly, *E. coli BL21(DE3)* bacteria transformed with GST-hBD-4-recombinant plasmid were induced by 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 proteases inhibitors), and sonicated using ultrasound disintegrator (UD-11 Automatic, Poland). Then cell lysate was applied to affine chromatography on glutathione-agarose column (GE Healthcare, Sweden) with following cleavage of the defensin from fusion protein by thrombin digestion. hBD-4 peptide was further purified by reverse phase chromatography on Sep-Pack C18 cartridge (Waters, USA). Protein concentration was determined by UV absorbance at 280 nm using spectrophotometer Nanodrop-1000 (USA), and purity of the peptide preparation was analyzed by 7–22% gradient SDS PAAG electrophoresis.

Direct cell counting. To study the effect of rec-hBD-4 on cell proliferation, A431, A549, and TPC-1 cells were routinely cultured in 24-well plates ($5x10^4$ cells per well) to nearly 50% confluence and then treated by addition of rec-hBD-4 at various concentrations (from 0.1 nM to 1 µM) for 48 h in 2.5% FBS medium. After the treatment, cells were triply 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-4 on cell viability, MTT-test has been applied [29]. A549, A431, and TPC-1 cells were seeded into 96-well plates ($7x10^3$ cells per well) and incubated with rec-hBD-4 for 48 h in medium with 2.5% FBS. Then the cells were routinely treated with MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) by standard protocol, and colorimetric reaction was evaluated with the use of ELISA reader (Awareness Technology Inc, USA) at $\lambda = 545$.

Flow cytometry analysis. Cells were cultured in 10 cm dishes in DMEM culture medium supplemented with 10% FBS to 50% confluency, then the medium was replaced by the fresh DMEM supplemented with 2.5% FBS, and the cells were treated by addition of 1; 10; 100; 250; 500; 750, or 1000 nM rec-hBD-4 for 48 h. After the treatment, the attached cells were triply washed with PBS, harvested by trypsinization, pelleted at 4 °C (500 g) for 5 min, washed twice in PBS, and re-

suspended in 1 ml of hypotonic cell lysis buffer (0.1% sodium citrate, 0.1% Triton X-100, 5 mg/ml PI (Sigma, USA)). The 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.

Antimicrobial activity of rec-hBD-4 was analyzed against *P. aeruginosa* by bacterial growth suppression in radial diffusion assay [30]. *P. aeruginosa* ATCC 9027 strain was obtained from Ukrainian Collection of Microorganisms (D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine, Kyiv, Ukraine).

Scratch wound healing assay. An effect of rec-hBD-4 on mobility of cultured human cancer cells was performed by scratch wound healing assay [31]. The cells were seeded in 6-well plates ($15x10^4$ cells per well) and routinely grown till 80% confluence, than a scratch was performed using a 20 µl tip, and the cells were grown for next 24 h in the presence of 100, 500 or 1000 nM rec-hBD-4 in serum free medium. Then the cell migration into wound area was examined using inverted microscope Axioplan (Zeiss, Germany).

Colony forming assay. Substrate independent growth was studied by routine procedure [32]. The cells were seeded in 3 cm Petri dishes ($5x10^3$ cells) in the medium containing 0.8% methylcellulose, 2x DMEM, 30% FBS and 100; 500, or 1000 nM rec-hBD-4, and were cultured in 5% CO_2 at 37 °C for 2 weeks. Then the colonies were stained with 0.01% neutral red in PBS for 30 min, and examined by light microscopy. TotalLab program has been used for data calculation.

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 AND DISCUSSION

hBD-4 cloning and prokaryotic expression of hBD-4-GST fusion protein. To clone the gene encoding mature hBD-4, a predicted 50 amino acid protein of 5.9 kDa, *hBD-4* cDNA was amplified from total RNA isolated from human lung squamous cell carcinoma sample No 5 [27]; in this particular tumor sample we have registered *hBD-4* up-regulation during the screening of hBDs expression in human lung tumors [27].

For expression of rec-hBD-4 we have used GST-system for production of recombinant proteins that allows protein expression in *E. coli* strains in a form of chimeric proteins fused to GST tag. Both PCR product and pGEX-2T vector were purified, digested by *BamHI* and *EcoRI* restriction endonucleases, ligated and the hybrid plasmid was used for subsequent transformation of *E. coli* BL21(DE3) cells. After selection of colonies, recombinant vectors were analyzed by digestion with *BamHI* and *EcoRI* endonucleases; the result evidenced correct 150 bp insertion (Fig. 1) which sequence was shown to correspond to nucleotide sequence of *hBD-4* gene by direct DNA sequencing analysis.

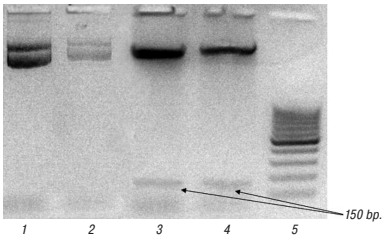

Fig. 1. Recombinant hBD-4-pGEX-2T construct digested with *BamH1* and *EcoRI* endonucleases. Lines 1, 2 — undigested plasmid; 3, 4 — recombinant plasmid digested with *BamH1* and *EcoRI* endonucleases; line 5 — DNA ladder (Fermentas, USA)

Purification of rec-hBD-4 has been performed by routine three-step procedure including affine chromatography, proteolysis of fusion protein with thrombin and reverse phase chromatography. In brief, *E. coli BL21DE3* cells transformed with hBD-4-pGEX-2T construct were induced with 1 mM IPTG for 6 h; the cells were collected and disrupted by sonication in lysis buffer containing 1% Triton X-100 and cocktail of protease inhibitors. Bacterial cell lysate was clarified by centrifugation, and supernatant was applied on glutathione-agarose column (GE Healthcare, Sweden); affine chromatography was performed by routine procedure according to instructons of the manufacturer. Then the fusion protein was treated with thrombin (1 U of thrombin per 1 mg of fusion protein), and hBD-4 peptide was purified from proteolytic mixture by reverse phase chromatography on Sep-Pak C18 column (Water, USA) in acetonitrile gradient. Pure hBD-4 fraction was eluted by 70–100% acetonitrile, collected, dried in Speed-Vac, analyzed by gradient 7–22% SDS-PAGE (Fig. 2) and by inhibitory zone test. Rec-hBD-4 was active against *P. aeruginosa* in a dose of 10–15 µM (data not shown). In summary, prokaryotic expression of hBD-4 and purification procedure allowed to purify up to 5 mg of biologically active rec-hBD-4 from 1 L of IPTG-induced bacterial culture.

Effect of rec-hBD-4 on proliferation and viability of human cancer cells in vitro. We have analyzed an effect of rec-hBD-4 on proliferation and viability of three human cancer cell lines — A431 (epidermoid carcinoma cells), A549 (lung adenocarcinoma cells), and TPC-1 (papillary thyroid cancer cells). As it has been shown by direct cell counting technique (Fig. 3), rec-hBD-4 exerts a concentration-dependent effect on the cell proliferation and stimulates cell proliferation in the concentrations from 1 to 100 nM and significantly suppresses — in concentrations ⩾ 500 nM. Effects of hBD-4 were similar in A431 and A549 cells but TPC-1 cells seems to be less sunsitive to low nanomolar concentration of rec-hB44 (Fig. 3). According to the data of trypan blue staining, the percentage of dead cells in all samples didn't exceed 4–5% for any hBD-4 concentration, so one may conclude that in nanomolar concentrations this defensin does not cause cell death.

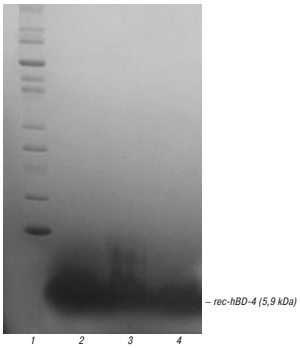

Fig. 2. SDS-PAGE analysis of rec-hBD-4 purified by reverse phase chromatography on Sep-Pak C18 (Water, USA). 1 — molecular weight marker (SM 0661, Fermentas, USA); fractions of rec-hBD-4 eluted with 80% CH_3CN (2), 70% CH_3CN (3), 100% CH_3CN (4)

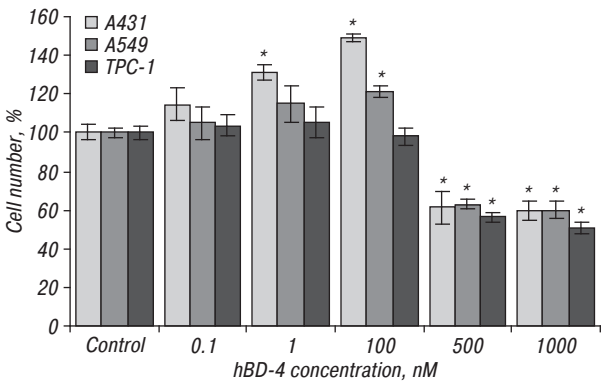

Fig. 3. A concentration-dependent effect of rec-hBD-4 on the number of viable cultured cells of A431, A549, and TPC-1 lines. The number of attached cells was evaluated by direct cell counting. Cells were cultured in 24-well plates ($5x10^4$ cells per well) to nearly 50% confluence, then culture medium was replaced with fresh DMEM supplemented with 2.5% FBS and rec-hBD-4 was added into cell cultivation medium in concentrations of 0.1; 1; 100; 500; 1000 nM, and the cells were cultured with rec-hBD-4 for 48 h. 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-4 on viability of cultured A431, A549 and TPC-1 cells using MTT assay. Our data have shown that in low concentrations (0.1–100 nM) rec-hBD-2 caused insignificant increase of viability of A431, TPC-1 and 549 cells (Fig. 4). In all three cell lines treated with 500 nM of rec-hBD-4, a decrease of cell viability has been recorded while treatment with 1 μM of rec-hBD-4 resulted in significant increase of cell viability (Fig. 4). In concentrations more than 1 μM rec-hBD-4 caused significant suppression of viability of A431, A549 and TPC-1 cells.

Interestingly, such multimodal effects of rec-hBD-4 on cell viability were found to be reflected in its effects on cell cycle distribution. When A431 cells treated with 1 nm — 1 μM rec-hBD-4 were subjected to flow cytofluorometry analysis, it has been revealed (Fig. 5) that treatment of the cells with 100 nM rec-hBD-4 resulted in significant stimulation of cell cycle in G2/M checkpoint, with 500 nM — in significant blockage of cell cycle in G1/S checkpoint; higher concentrations of hBD-4 insignificantly suppressed cell cycle progression (Fig. 5). Similar results were obtained with A549 and TPC-1 cells treated with rec-hBD-4 (data not shown).

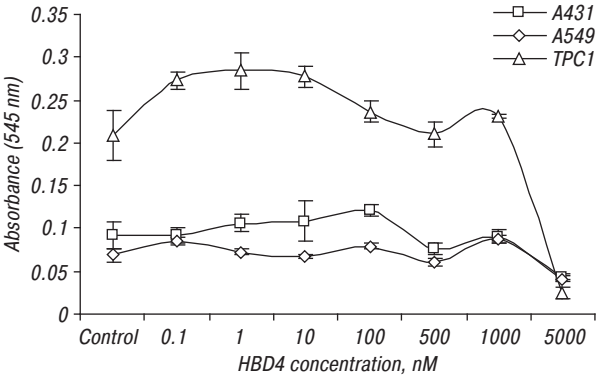

Fig. 4. A concentration-dependent effect of rec-hBD-4 on viability of A431, A549, and TPC-1 cells. Cells were cultured in 96-well plates ($7x10^3$ cells per well) and incubated with rec-hBD-4 in concentrations from 100 pM to 5 μM for 48 h in medium supplemented with 2.5% FBS. The number of viable cells was evaluated by MTT analysis. The data of three independent experiments are presented as the mean ± SD

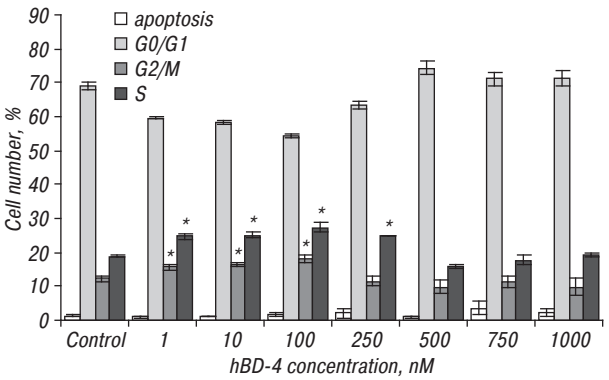

Fig. 5. Flow cytofluorometry analysis of cell cycle distribution of A431 cells treated with rec-hBD-4 for 48 h. Cells were cultured in 10 cm dishes in culture medium supplemented with 10% FBS to 50% confluency, then the medium was replaced by fresh DMEM supplemented with 2.5% FBS, and cells were treated by addition of 1; 10; 100; 250; 500; 750, or 1000 nM rec-hBD-4 for 48 h. After the treatment, the cells were triply washed with PBS, detached with trypsin and subjected to flow cytofluorometry analysis as described in Materials and Methods section. The data of two independent experiments are presented as the mean ± SD.
*The difference is significant as compared to appropriate control ($p < 0.05$)

Rec-hBD-4 affects colony-forming activity of epithelial cancer cells. We have performed colony forming assay to evaluate possible effects of rec-hBD-4 on important biological property of cancer cells — their ability for substrate independent growth. In this experiment we have used three concentrations of hBD-4 (100, 500, 1000 nM); addition of the defensin into cell incubation medium significantly affected A431cell ability to form colonies in semi-soft medium (Fig. 6): treatment with 100 nM rec-hBD-4 resulted in significant increase of colony numbers compared to control untreated cells ($p < 0.05$), with 500 nM — in significant decrease of colony counts ($p < 0.05$), while in the presence of 1 μM of the defensin no visible colonies were developed. Similar

results were obtained with A549 cells treated with rec-hBD-4 (data not shown).

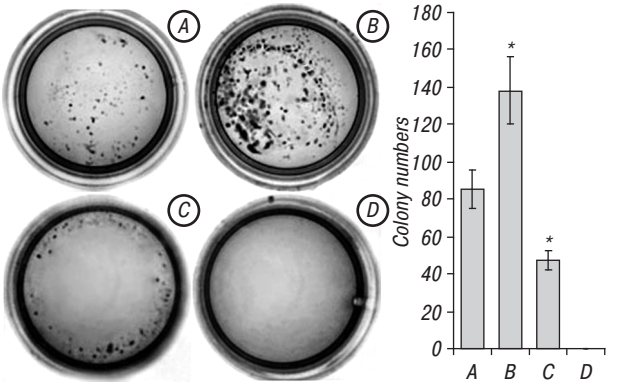

Fig. 6. Rec-hBD-4 significantly affects colony-forming activity of A431 cells. The cells ($5x10^3$) were cultured in 3 cm Petri dishes ($5x10^3$ cells) in the medium containing 0.8% methylcellulose, 2x DMEM, 30% FBS in the absence (control cells, *A*) or presence of rec-hBD-4 (100 nM (*B*), 500 nM (*C*) or 1 µM (*D*)). When visible colonies developed the plates were stained with 0.01% neutral red. The graph represents the numbers of colonies calculated with the use of TotalLab program.
*The difference is significant as compared to appropriate control ($p < 0.05$)

Effect of rec-hBD-4 on cancer cell migration ability. To analyze a possible effect of rec-hBD-4 on cancer cell mobility, wound healing assay has been applied. A431 and TPC-1 cells were seeded in 6-well plates ($15x10^4$ cells per well) and routinely grown till 80% confluence, then a scratch was performed using a 20 µL tip, and the cells were grown for next 48 h in the presence of 100; 500 or 1000 nM of rec-hBD-4 in serum free medium. Light microscopy examination has revealed (Fig. 7) that 500 nM of rec-hBD-4 significantly suppressed migration of TPC-1 cells while treatment with 100 or 1000 nM of rec-hBD-4 seems to have no notable effect on TPC-1 cell mobility. In A431 cell line defensin moderately affected cell migration: stimulation of cell mobility has been detected in cells treated with 100 nM hBD-4 but 500 or 1000 nM of the defensin had insignificant influence on migration of A431 cells (Fig. 7).

DISCUSSION

Antimicrobial peptides in general and defensins in particular are in spite of scientific interest in recent years due to the search for new antibiotics which could be used against highly resistant clinical isolates. Really, defensins as natural compounds with potent antimicrobial activity could be successfully used to kill even antibiotic-resistant pathogens [33]. Apart from this, defensins may be potentially used in other fields of medicine, for example, as anticancer or wound-healing agents [13, 34].

For experimental purposes defensins are produced as synthetic peptides or recombinant molecules expressed in prokaryotes. In the latest case, to avoid host cell autodestruction and to simplify purification procedures, defensins are cloned and expressed in a form of fusion proteins containing His-Tag, GST, TrxA, or other tags. Such approach has allowed to produce biologically active recombinant defensins — hBD-3 [9], hBD-2 [29], hBD-26 and -27 [35], and some others. Similarly, in present work we have produced soluble biologically active recombinant hBD-4 expressed in prokaryotic system as GST-hBD-4 fusion peptide and for the first time characterized its *in vitro* activity toward human cancer cells.

Up to date, just few articles have described the biologic activity of hBD-4 [8, 36]. In these studies synthetic peptide has been used; it has been demonstrated that hBD-4 is active against *P. aeruginosa* and *Staphylococcus carnosus*, and its antimicrobial activity depends on salt concentration; hBD-4 is chemotactic for human blood monocytes at the concentration of 10 nM, and its chemotactic activity is independent from intracellular calcium mobilization [8]. hBD-4 has been supposed to play an important role in defense of low respiratory tract against infections, especially those caused by *P. aeruginosa* [36], and in *H. pylori* associated gastritis [37].

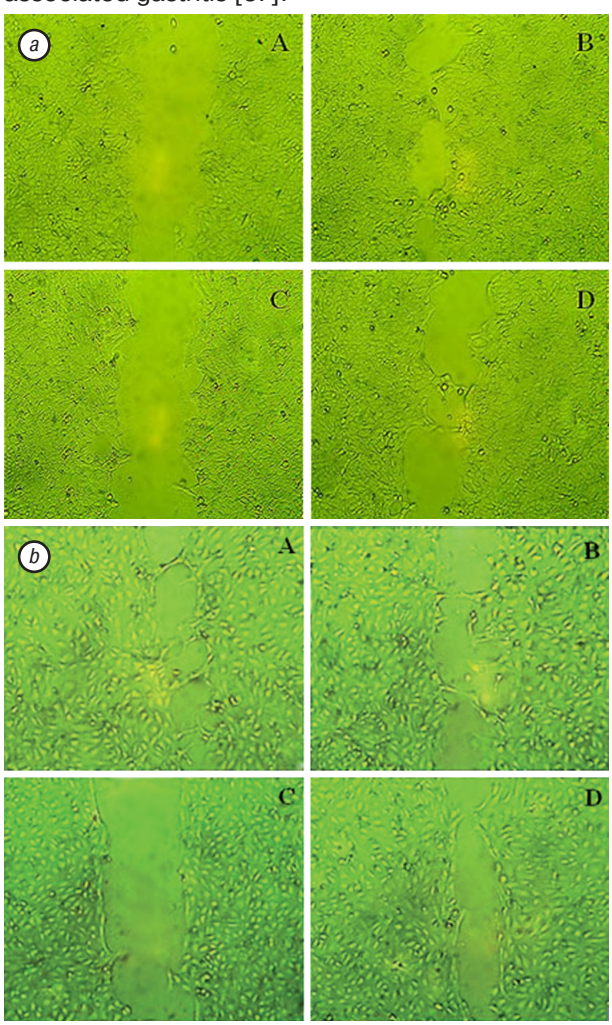

Fig. 7. Wound healing assay in A431 (*a*) and TPC-1 (*b*) cells treated with 100 nM (B), 500 nM (C) or 1000 nM (D) rec-hBD-4 for 48 h. A — control untreated cells. The results of typical experiment are presented

Our research has demonstrated for the first time that hBD-4 could exert a concentration dependent effect on biological properties of cultured human cancer cells: in low nanomolar concentrations (1–100 nM) rec-

hBD-4 stimulates cancer cell proliferation and viability, promotes cell cycle progression through G2/M checkpoint, significantly enhances colony-forming activity and mobility of A431, A549, and TPC-1 cells. Treatment of cells with 500 nM of rec-hBD-2 resulted in opposite effects: significant suppression of cell proliferation and viability, blockage of cell cycle in G1/S checkpoint, significant inhibition of cell migration and colony forming activity. In concentrations more than 5 μM hBD-4 is cytotoxic to all studied cell lines. It's necessary to note that biologic effects of rec-hBD-4 were similar, but not equal in all three studied cell lines: for example, thyroid papillary carcinoma cell migration ability is more sensitive to hBD-4 than that of A431 cells.

So, biologic effects of nanomolar concentrations of rec-hBD-4 are of bimodal character, unlike to these of recombinant hBD-2 described in our earlier publication [21]. So, hypothetically, at low nanomolar range human beta-defensin-4 may play a role of pro-oncogenic molecule via stimulation of cell proliferation and cell malignancy potential, and in high nanomolar range hBD-4 could play a role in tumor suppression. It's of interest to further explore the mechanisms of regulation of hBD-4 expression in human epithelial cells, patterns of its expression in human carcinoma *versus* normal tissue, and its physiologic content in biologic fluids and tissues to understand the possible role of this defensin in tumorigenesis.

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