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Effects of RHAMM/HMMR-Selective Peptides on Survival of Breast Cancer Cells

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RHAMM-selective peptides in a concentration of 10 μg/ml (2×10^{-7} M) inhibited the growth of MDA-MB-231 breast cancer cells over 48 h. Treatment of cancer cells with RHAMM-selective peptides induced apoptosis and necrosis and increased caspase-3 activity (by 30%).

Key Words: *RHAMM-selective peptides; RHAMM/HMMR (hyaluronan-mediated motility receptor); apoptosis; necrosis; breast cancer*

Hyaluronan-mediated motility receptor (HMMR) is a multifunctional extra- and intracellular protein that utilizes hyaluronic acid (HA) as the substrate and interacts with tubulin [2] involved in the formation of the mitotic spindle [7]. HA is a polysaccharide with structural and signal functions in the cell, and hence, HA is involved in immunity, tissue differentiation, and homeostasis [5]. The function of HA depends on the polysaccharide size: high-molecular-weight HA is responsible for structural functions, low-molecular-weight HA binds to and interacts with cell receptors (CD44 and RHAMM). These low-molecular-weight fragments of HA transmit signals to cell pathways regulating proliferation, differentiation, adhesion, motility, and invasive activity of cancer cells [6,12,14]. Excessive synthesis of high-molecular-weight HA is observed in arthritis, diabetes, and cancer [1,14]. RHAMM is involved in proliferation, migration, invasion, and formation of the mitotic spindle in tumor cells [4,13,14]. It is shown that RHAMM is synthesized in excess in aggressive cancer cells, such as BC cells, hematological, prostatic, colorectal cancer cells, in solid tumors, myeloid leukemia, and multiple myeloma. Enhanced synthesis of RHAMM and HA usually correlates with poor prognosis [3,8]. It is shown on model cancer systems that carboxyl-terminal RHAMM sequence contains binding center for HA, tubulin, and special sites essential for manifestations of its oncogenic characteristics [9]. For these reasons, RHAMM is an ideal molecular target for the development of targeted antitumor drugs. Peptides specifically interacting with RHAMM have been identified and characterized [10,15]. It is shown that RHAMM-selective peptides compete for HA binding center, selectively bind recombinant RHAMM protein, easily penetrate into cancer cells, and are stable in blood serum [10]. However, the effects of RHAMM-selective peptides on the growth of BC cells are not studied.

We studied the effects of RHAMM-selective peptides on survival, apoptosis, and necrosis of BC cells *in vitro* in cell cultures.

MATERIALS AND METHODS

The study was carried out on three cell lines: human mammary carcinoma adhesive cells MDA-MB-231 with high expression of RHAMM, normal mouse fibroblasts, and RHAMM$^{-/-}$ fibroblasts. The RHAMM$^{-/-}$ fibroblasts cell lines were obtained as described previously [11]. Adhesive culturing was carried out in DMEM with 10% fetal calf serum at 37°C and 5% CO_2.

RHAMM-selective peptides (EEDFGEEAEEEA – peptide No. 35; VEGEGEEGEEY – peptide No. 37; and FTEAESNMNDLV – peptide No. 40) were obtained as described previously [10,15].

The studied cell lines were inoculated in flat-bottom 96-well plates (1000 cells/well) and cultured

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for 24 h in an incubator at 37°C, 5% CO_2, and 95% humidity. Growth medium was added to all cell lines. MDA-MB-231 cells were cultured in 200 µl growth medium under the same conditions. RHAMM-selective peptides in a concentration of 10 µg/ml (4 µM) were then added to the wells (100 µl/well) in order to evaluate cell survival or the effects of peptides on apoptosis and necrosis, respectively, and the plates were incubated for 24 h at 37°C. An equal volume of PBS was added into control wells. All measurements were repeated 3 times.

Cell survival was evaluated by the fluorescent method (AlamarBlue) based on evaluation of transformation of the "blue" slightly fluorescent reazurin into "pink" intensely fluorescent resofurin by the living cell mitochondrial dehydrogenase. AlamarBlue (10 µl) was added directly to MDA-MB-231 cells into growth medium after 0.5, 3, 24, and 48 h. The fluorescence intensity was measured at 570/590 nm using a fluorescent plate. Cell survival under the effect of RHAMM-selective peptides was evaluated by comparing the fluorescence in experimental and control wells. The results were presented as the means of 3 independent experiments.

Quantitative evaluation of the peptide effects on apoptosis and necrosis of cells was carried out using ELISA^{PLUS} Kit (Roche Diagnostic). Apoptosis and necrosis induction were evaluated by measuring histon components of mono-, oligonucleosomes (histons H1, H2A, H3, and H4). Optical density was measured at 405/490 nm. All measurements and all experiments were carried out in triplicates.

Caspase-3 activity was measured by colorimetry. Human BC cells were inoculated in 24-well plates (1000 cells/well) and incubated for 24 h in DMEM (Multicell) with 10% fetal calf serum, after which RHAMM-selective peptides were added (4 µl, 100 µl/well) to the cells and incubated (24 h, 37°C). Control cultures were incubated in growth medium with 10% serum. Caspase-3 activity was measured with Caspase-3 Colorimetric Assay Kit (GenScript). Activation of caspase-3 was evaluated by cleavage of the caspase-3-specific colorimetric substrate (DEVD-p-nitroanilide). Optical density of each well was measured using a Wallac 1420 multirow counter (Perkin Elmer) at 405 nm. The data were presented as the means of 3 repeated experiments.

The data were statistically processed by GraphPadPrizm software (one-way ANOVA). The differences were considered significant at $p<0.05$.

RESULTS

All three peptides inhibited the growth of tumor cell cultures (Fig. 1, *a*). Incubation with the peptides for 48 h suppressed cell viability by 50%. It should be noted that viability of BC cell cultures was effectively suppressed by RHAMM inhibitors in low concentrations, a true advantage of these peptides as prospective antitumor drugs.

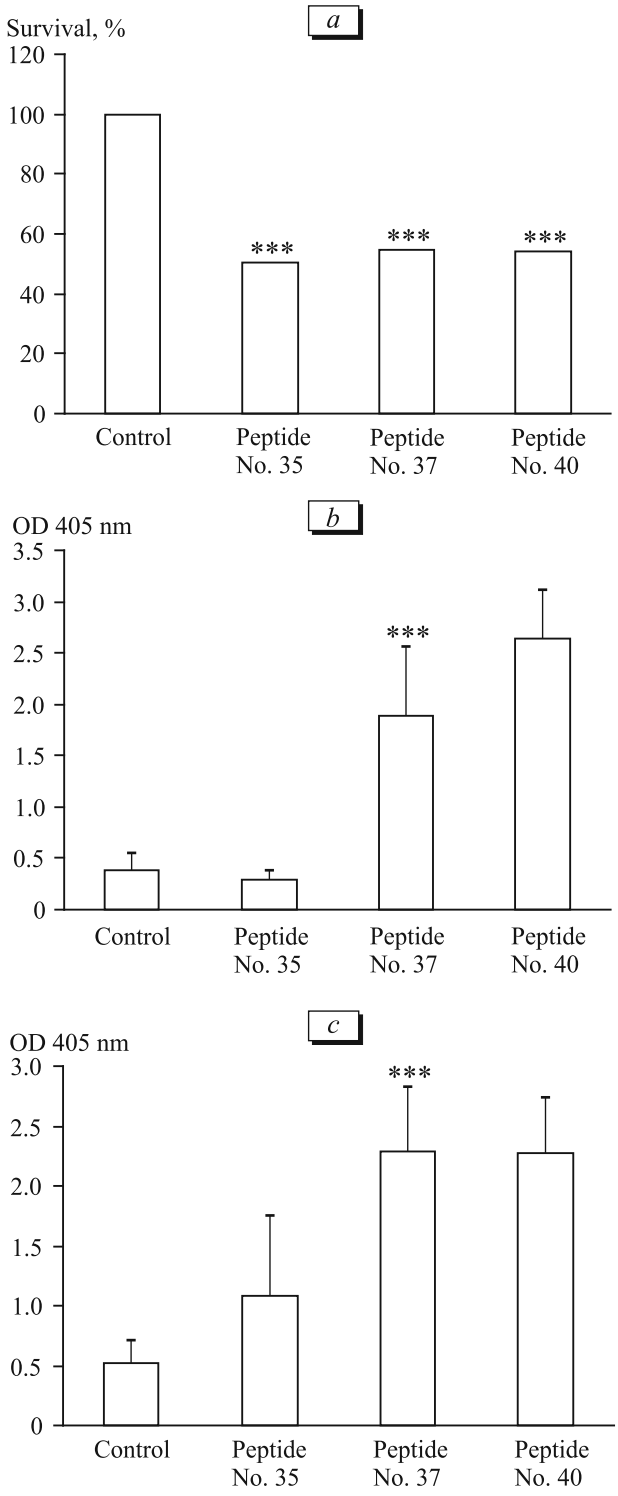

Fig. 1. Effects of RHAMM-selective peptides on MDA-MB-231 cell survival (*a*), apoptosis (*b*), and necrosis (*c*). Here and in Fig. 3: ***$p<0.001$ in comparison with the control.

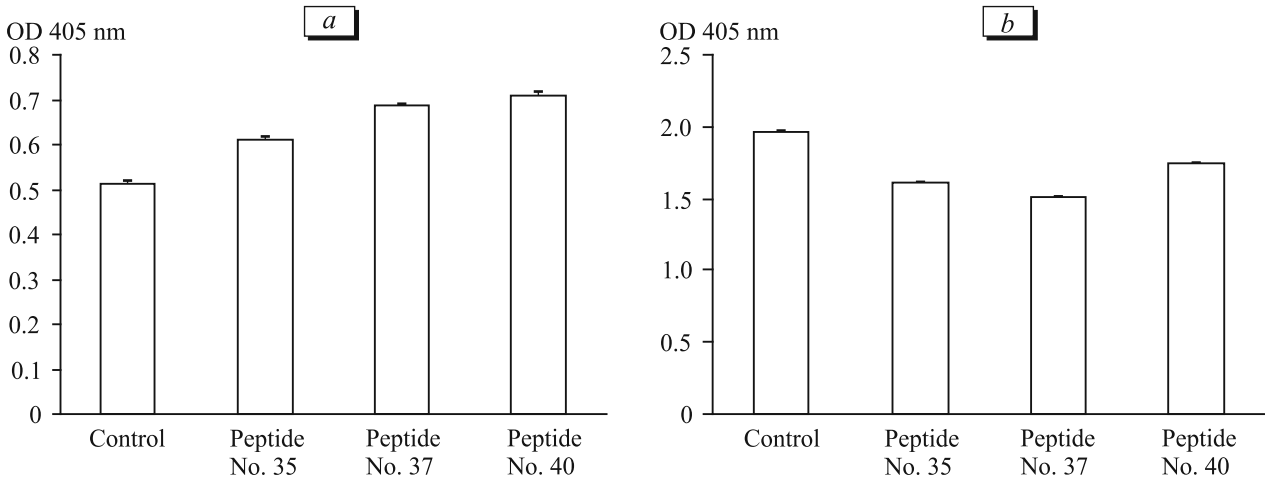

Fig. 2. Effects of RHAMM-selective peptides on fibroblast apoptosis (*a*) and necrosis (*b*).

As cell survival was significantly reduced by RHAMM-selective peptides, it was essential to identify the MDA-MB-231 cell death type. RHAMM inhibitors in a concentration of 10 μg/ml (2×10^{-7} M), especially peptides Nos. 37 and 40, 4-5-fold increased apoptosis induction in the cells (Fig. 1, *b*). These results suggest that RHAMM inhibitors were involved in apoptosis pathways in MDA-MB-231 cells. Necrotic death of MDA-MB-231 cells was increased significantly (approximately 4-fold) by peptides Nos. 37 and 40 (Fig. 1, *c*). In order to verify the selective effects of the peptides on tumor cells, we studied the effects

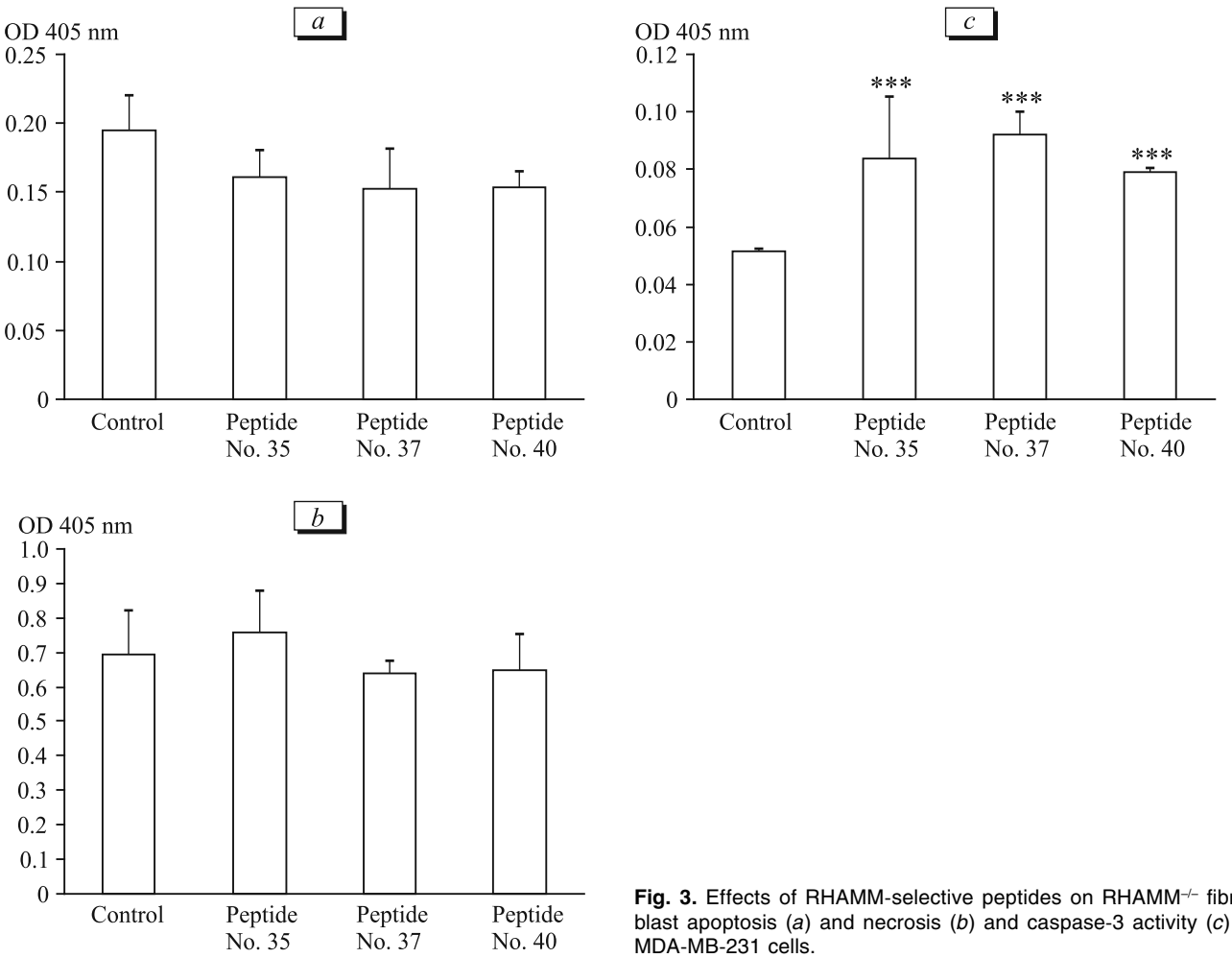

Fig. 3. Effects of RHAMM-selective peptides on RHAMM$^{-/-}$ fibroblast apoptosis (*a*) and necrosis (*b*) and caspase-3 activity (*c*) in MDA-MB-231 cells.

of RHAMM inhibitors on apoptosis and necrosis of normal fibroblasts. RHAMM-selective peptides caused virtually no changes in apoptosis and necrosis of normal cells (Fig. 2, *a*, *b*). All three peptides caused in fact no changes in fibroblast ($RHAMM^{-/-}$) apoptosis and necrosis (Fig. 3, *a*, *b*). These results confirmed that the effects of RHAMM-selective peptides were tumor-specific and RHAMM-mediated. These findings suggest that RHAMM-selective peptides in low concentrations induced death of breast cancer cells (MDA-MB-231) within 24 h after their addition.

Activity of caspase-3 increased by 30% in MDA-MB-231 cells in comparison with intact cells (Fig. 3, *c*). These data confirmed the involvement of caspase-3 in cancer cell apoptosis induction, as all three peptides stimulated significantly its activity.

Hence, RHAMM-selective peptides inhibit the viability and induce apoptosis and necrosis of breast cancer cells. These data suggest the use of RHAMM-selective peptides for target therapy for cancer, as they are tumor cell-specific and easily synthesized.

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