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The effect of *Galleria mellonella* hemolymph polypeptides on human brain glioblastoma multiforme cell line – a preliminary study

Badania wstępne wpływu polipeptydów hemolimfy *Galleria mellonella* na komórki ludzkiego glejaka wielopostaciowego

SUMMARY

Anticancer activity of hemolymph of naive and immune-challenged *Galleria mellonella* larvae was tested on human brain glioblastoma multiforme cell line T98G. As revealed by Hoechst 33342 and propidium iodide staining, the hemolymph and methanolic extracts of hemolymph containing peptides and proteins below 30 kDa, induced apoptosis and/or necrosis of the T98G cells. It was detected that treatment of the cells with hemolymph of immunized insects, enriched with inducible antimicrobial peptides, increased three times the number of the dead cells in comparison with the control cultures. To our knowledge it is the first report addressing investigations on anticancer activity of *G. mellonella* hemolymph against human brain cancer cells.

Keywords: *Galleria mellonella*, hemolymph, apoptosis, necrosis

STRESZCZENIE

Przebadano wpływ hemolimfy nieimmunizowanych i immunizowanych gąsienic barciaka większego *Galleria mellonella* na komórki nowotworowe linii ludzkiego glejaka wielopostaciowego T98G. Stosując barwienie fluorochromami Hoechst 33342 i jodkiem propidyny, wykazano, że hemolimfa oraz metanolowe ekstrakty hemolimfy zawierające peptydy i białka o masie cząsteczkowej poniżej 30 kDa, indukowały apoptozę i/lub nekrozę komórek T98G. Inkubacja komórek T98G w obecności hemolimfy gąsienic immunizowanych, wzbogaconej w indukowalne peptydy odpornościowe, powodowała trzykrotny wzrost liczby martwych komórek w porównaniu z komórkami w kulturze kontrolnej. Prezentowane wyniki stanowią pierwsze doniesienie dotyczące aktywności hemolimfy *G. mellonella* w stosunku do komórek nowotworowych tkanki nerwowej mózgu człowieka.

Słowa kluczowe: *Galleria mellonella*, hemolimfa, apoptoza, nekroza

INTRODUCTION

Antimicrobial peptides are important factors of the innate immunity in a wide range of organisms. They are small, usually cationic, amphipathic molecules. According to their amino acid sequence and biochemical properties they are divided into three classes: (i) linear peptides without cysteine, e.g. cecropins, (ii) peptides whose structure is stabilized by disulfide bridges, e.g. insect defensins, (iii) peptides with overrepresentation of one amino acid. Several models explaining cationic peptide mode of action against microbial targets were proposed: (i) carpet-like model, (ii) barrel-stave model, (iii) toroidal pore model, (iv) aggregation model. Most peptides act on the level of pathogen cell membrane leading to channel formation, increased permeabilization, fragmentation and eventually to cell death. Some of them traverse cell membrane and once inside a cell influence replication, transcription, translation and proper folding of proteins (3, 10, 11, 15, 26, 30, 31).

It was demonstrated that some antimicrobial peptides, besides antibacterial, antifungal or antiviral activity, exhibited also anticancer activity. The majority of these peptides adopt α-helical structure. It was suggested that they kill cancer cells by carpet-like and/or toroidal pore model of action leading to loss of membrane integrity, apoptosis and necrosis. The well characterized anticancer peptides include amphibian magainins, aurein, horseshoe crab tachyplesin, honeybee melittin (9, 19, 23, 28). Different studies demonstrated specific tumoricidal activity of insect cecropin A and B against mammalian leukaemia, lymphoma, colon carcinoma cell lines (6, 22), small cell lung cancer (27), gastric cancer cells (5), as well as bladder cancer cell line (29).

In insects antimicrobial peptides are synthesized mainly in a fat body (functional analogue of mammalian liver) and are released into hemolymph (insect blood) where they play crucial role in the fight against invading pathogens. Hemolymph of the lepidopteran greater wax moth *Galleria mellonella* is a very rich source of antimicrobials, as it contains defence peptides with different biochemical and antimicrobial properties (2, 7, 16, 18, 20).

In the present study we investigated a potential anticancer activity of *G. mellonella* hemolymph proteins and peptides using human brain glioblastoma multiforme cells. Gioblastoma multiforme is the most common and the most aggressive of malignant glial tumors. Glioblastoma therapy can involve chemotherapy, radiotherapy and surgery but none of them is effective enough, the patients survive usually only 1–2 years. Thus it is necessary to search for a new approach in glioblastoma treatment (13). To our knowledge, it is the first paper in which anticancer activity of *G. mellonella* hemolymph against brain tumor cells was evaluated.

MATERIALS AND METHODS

Insect culture, immune-challenge and hemolymph collection

Larvae of the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) were reared on honey bee nest debris at 28°C in the dark. Last instar (seventh) larvae were used throughout the study.

Immune challenge was performed by piercing of the larval abdomen with a needle dipped in a pellet of *E. coli* D31 cells. The insects were kept at 28°C in the dark and the hemolymph (20 µl per larvae) was collected 24 hrs. after a challenge. Prior to hemolymph collection, the insects were chilled for 15 min. at 4°C. The hemolymph samples were obtained by puncturing of larval abdomen with a sterile needle. Out-flowing hemolymph was immediately transferred into sterile and chilled Eppendorf tubes containing a few crystals of phenylthiourea (PTU) to prevent melanisation. The hemocyte-free hemolymph was obtained by centrifugation at 200×*g* for 5 minutes and subsequently at 20000×*g* for 15 min. at 4°C. Pooled supernatants were used immediately or stored at -20°C until used.

Preparation of methanolic extract of *G. mellonella* hemolymph

Methanolic extracts of hemolymph containing proteins of molecular mass below 30 kDa and defence peptides were obtained from the hemocyte-free hemolymph as described previously (7). Briefly, the hemolymph proteins of molecular mass higher than 30 kDa were precipitated from the hemolymph ten times diluted with the extraction solution consisting of methanol/acetic acid, glacial/water (90:1:9) and were pelleted by centrifugation at 20000×*g* for 30 min. at 4°C. The supernatant containing the defence peptides was collected, vacuum dried and the pellet was stored at -20°C until needed.

Antimicrobial activity assays

Antibacterial activity assay

Antibacterial activity of the hemolymph and the hemolymph methanolic extracts was detected by a diffusion well assay using solid agar plates containing viable *E. coli* D31 cells and hen egg white lysozyme (EWL) in the concentration of 2.5 mg/ml of the medium as described previously (8). Each well on the Petri dish was filled with 5 µl of hemolymph four times diluted with sterile water or 5 µl (10 µg of protein) of dissolved partially purified peptides. The agar plates were then incubated at 37°C for 24 hrs. The diameters of *E. coli* D31 growth inhibition zones were measured. The level of antimicrobial activity was calculated using the algorithm described by Hultmark et al. (12). For evaluation of antibacterial activity synthetic cecropin B of *Hyalophora cecropia* (Sigma) was used as a standard.

Antifungal activity assay

Antifungal activity was detected by a diffusion well assay against *Aspergillus niger* 71 using PDA plates (5% potato extract, 0.5 % dextrose, 0.7% agar, w/v) containing conidial spores (2.5×10^5 spores/ml of the medium). After filling of wells with 5 µl of hemolymph or 5 µl (10 µg of protein) of dissolved partially purified peptides, the plates were incubated for 24 hrs. at 28°C. Then the diameters of *A. niger* growth inhibition zones were measured. The level of antifungal activity was calculated on the basis of standard curve prepared for known concentrations of amphotericin B (21).

Cells and culture conditions

The human brain glioblastoma multiforme cells (T98G, IV grade according to WHO; ECACC 92090213) were grown in 3:1 mixture of DMEM and Nutrient mixture F-12 Ham (Ham's F-12) (Sigma) supplemented with 10% FBS (Sigma), penicillin (100 units/ml) (Sigma) and streptomycin

(100 μg/ml) (Sigma). The cultures were kept at 37°C in humidified atmosphere of 95% air and 5% CO_2.

For the experiments, the cells at a density 1 x 10^6 were incubated in 500 μl of the medium without (control) and in the presence of hemocyte-free hemolymph or the hemolymph extracts (final concentration of total protein 0.1–1.0 mg/ml and 0.064–0.2 mg/ml, respectively) for 24 hrs. at above described conditions. Then the level of apoptosis and necrosis was evaluated.

Apoptosis and necrosis detection with fluorochromes

For apoptosis and necrosis identification the cells were stained with fluorescent dye Hoechst 33342 (Sigma) and propidium iodide (Sigma), respectively (14). The morphological analysis was performed under a fluorescence microscope (Nikon E – 800). Cells exhibiting blue fluorescent nuclei (fragmented and/or with condensed chromatin) were interpreted as apoptotic. Cells exhibiting pink fluorescent nuclei were interpreted as necrotic. At least 1,000 cells in randomly selected microscopic fields were counted under the microscope. Each experiment was performed in triplicate.

Other techniques

Protein concentration was performed by the Bradford method using bovine serum albumin as a standard (1).

The proteins and peptides of hemolymph and methanolic extracts of hemolymph were resolved in 13.8% and 16.5% polyacrylamide gels by Tris-glycine and Tris-tricine SDS/PAGE, respectively (17, 24).

RESULTS AND DISCUSSION

Antimicrobial activity of *G. mellonella* hemolymph

Antibacterial and antifungal activity level of hemolymph of naive and immune-challenged *G. mellonella* larvae was measured using diffusion well assay against *E. coli* and *A. niger*, respectively. For this purpose, cell-free hemolymph as well as hemolymph methanolic extracts containing peptides and proteins below 30 kDa were tested. Antibacterial activity, calculated as 3.5 μM and 0.9 μM (cecropin B equivalent), was detected in hemolymph and the hemolymph extracts of challenged *G. mellonella* larvae, respectively. Similarly, antifungal activity of the immune hemolymph and hemolymph extracts was estimated as 155 μM and 95 μM (amphotericin B equivalent), respectively. Antimicrobial activity was not detected in the non-immune hemolymph and the corresponding extracts (Fig. 1). As demonstrated by SDS/PAGE, the antimicrobial activity of immune-hemolymph was correlated with the appearance of additional proteins and peptides (Fig. 2). Our earlier study revealed that the detected additional peptide bands corresponded to *G. mellonella* inducible defence peptides. From the extracts of *G. mellonella* immune hemolymph using RF-HPLC chromatography and gel filtration eight peptides differing in biochemical and antimicrobial properties were isolated, namely cecropin D, two defensins, two proline-rich peptides, two anionic peptides and apolipophoricin (7).

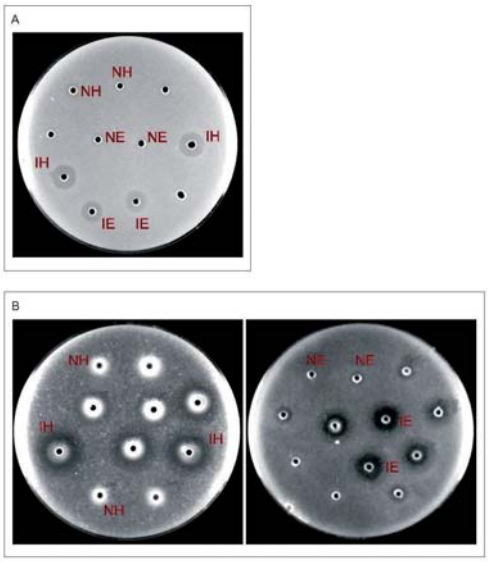

Fig. 1. Antibacterial (A) and antifungal (B) activity of *G. mellonella* hemolymph and the methanolic extracts of hemolymph. The samples (4 µl) of non-immune (NH) and immune (IH) hemolymph and the corresponding extracts (NE and IE, respectively) were tested, using diffusion well assay against *E. coli* (A) and *A. niger* (B). After 24 hrs. incubation the zones of microorganism growth inhibition were observed

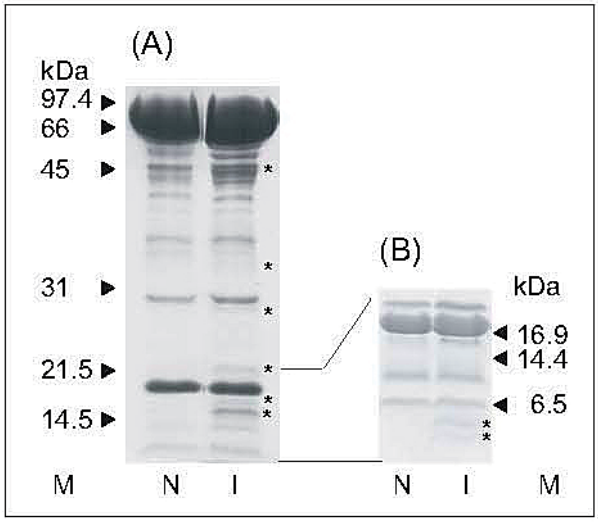

Fig. 2. Electrophoretic analysis of the proteins (A) and peptides (B) of non-immune (N) and immune (I) *G. mellonella* hemolymph (A) and the methanolic extracts of the hemolymph (B). The samples of the hemolymph (50 µg of total protein) and the methanolic extracts (20 µg of total protein) were resolved by 13.8% Tris-glycine SDS/PAGE (A) and 16.5% Tris-tricine SDS/PAGE (B), respectively. The asterisks indicate additional protein and peptide bands appearing in the hemolymph of immune-challenged insects. M – molecular weight markers

Analysis of the effect of *G. mellonella* hemolymph polypeptides on glioblastoma multiforme cells

The human brain glioblastoma multiforme cells were incubated in the presence of hemocyte-free hemolymph or the hemolymph extracts for 24 hrs. and then the level of apoptosis and necrosis was evaluated. Treatment of the cells with *G. mellonella* polypeptides resulted in induction of cell death, as some cells exhibited symptoms of apoptosis or necrosis as demonstrated in Figure 3. It was detected that non-immune as well as immune hemolymph increase percent of apoptosis and necrosis of T98G cells in comparison with the control ones

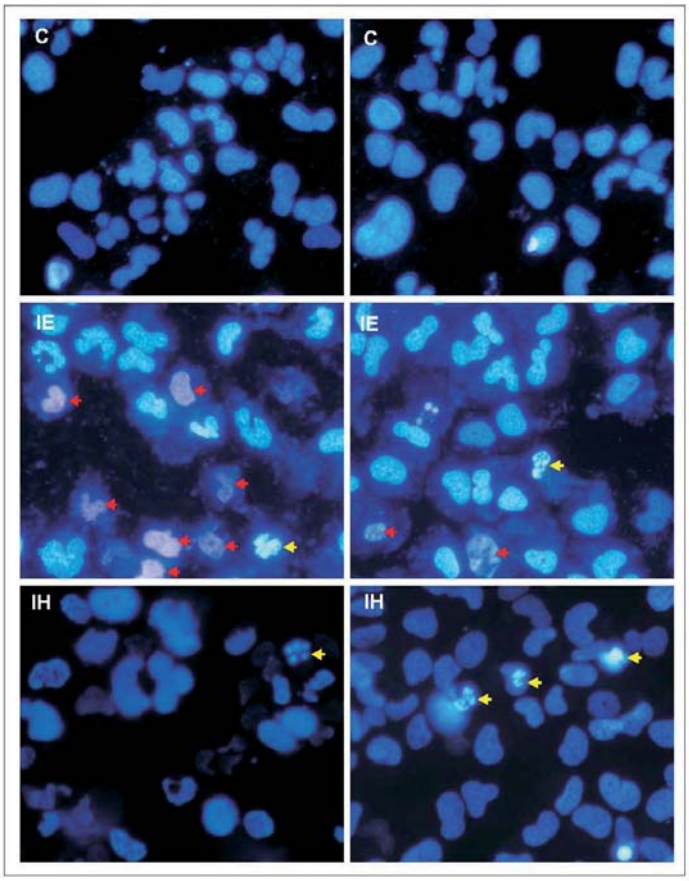

Fig. 3. Detection of apoptosis and necrosis of human brain glioblastoma multiforme cells after treatment with the polypeptides of *G. mellonella* hemolymph. The T98G cells were incubated without (control, C) and in the presence of immune hemolymph (IH, the final concentration of total protein 0.1 mg/ml) or corresponding extracts (IE, the final concentration of total protein 0.064 mg/ml) as described in Materials and Methods. Then apoptotic and necrotic cells were detected by fluorescent staining, using Hoechst 33342 and propidium iodide, respectively. The yellow and red arrows indicate the apoptotic and necrotic cells, respectively

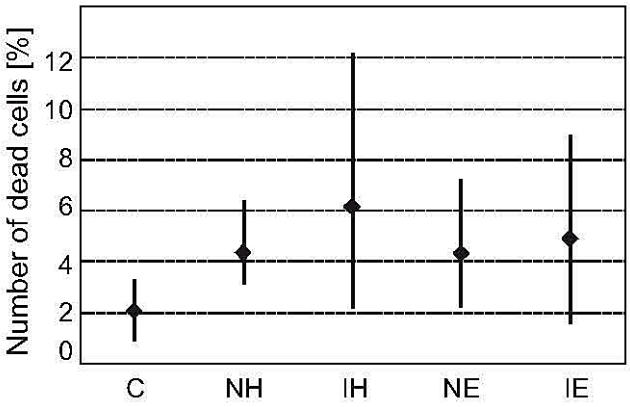

Fig. 4. The level of dead human brain glioblastoma multiforme cells after treatment with the polypeptides of *G. mellonella* hemolymph. The T98G cells were incubated without (control, C) and in the presence of non-immune (NH) or immune hemolymph (IH) and non-immune hemolymph extract (NE) or immune hemolymph extract (IE) as described in Materials and Methods. Then the apoptotic and necrotic cells were detected by fluorescent staining and counted under the microscope. The diagram presents the results of three independent experiments ±S.D

Table 1. The effect of *Galleria mellonella* hemolymph polypeptides on human brain glioblastoma multiforme cells

Tested compound (final concentration of total protein mg/ml)	Apoptosis (%)	Necrosis (%)	Dead (%)	Percent of dead cells in comparison with control
Control cells	0.94	1.11	2.04	100.00
Immune hemolymph (0.1 mg/ml)	3.21	2.93	6.14	300.98
Non-immune hemolymph (0.1 mg/ml)	2.07	2.27	4.33	212.25
Extract of immune hemolymph (0.064 mg/ml)	2.20	2.66	4.86	238.24
Extract of non-immune hemolymph (0.064 mg/ml)	3.17	1.13	4.30	210.78

(Fig. 4, Tab. 1). Non-immune and immune *G. mellonella* hemolymph used in the concentration of 0.1 mg/ml (of total protein) increased the level of the dead cells twice and three times, respectively. However, treatment of the cells with the hemolymph of immune-challenged insects resulted in higher percent of apoptotic cells (3.21% and 2.07% for immune and non-immune hemolymph, respectively). Higher concentrations of the hemolymph total protein, 0.5 mg/ml and 1.0 mg/ml, did not increase further the level of apoptosis and necrosis (not shown). Our

results suggested that the hemolymph of naïve *G. mellonella* larvae contained the factors inducing apoptosis of the cancer cells, however immune challenge of the insects induced an increase of the factors in the hemolymph and/or immunization caused appearance of additional compounds of such activity. The protein and peptide patterns of the immune hemolymph and corresponding extracts after SDS/PAGE analysis presented in Figure 2 could indicate for the second possibility.

The methanolic extracts of *G. mellonella* hemolymph increased the number of the dead cells about twice in comparison with the control samples (Fig. 4, Tab. 1). However, the non-immune hemolymph extract was more effective in inducing apoptosis than that of the immune hemolymph, increasing the apoptosis level by 3.37 and 2.34 times, respectively, comparing with the control samples. In contrast, the extract of the immune hemolymph increased 2.4 times the percent of the necrotic T98G cells, whereas that of non-immune hemolymph did not change this parameter (Tab. 1).

Cytotoxic activity of *G. mellonella* hemolymph against human tumor cell lines, K562 and Raji, and murine tumor cell line, L929, was reported by Chadwick and Aston (4). The activity was detected in non-immune and immune hemolymph, however the highest level of the activity was noticed in hemolymph obtained 2 hrs. after challenge of *G. mellonella* larvae by *Salmonella typhimurium* or *Pseudomonas aeruginosa* LPS. The cytotoxic activity was ascribed to *Gal*lysin 2, a wax moth hemolymph lysin, exhibiting TNF-like properties. However, the pro-apoptotic and pro-necrotic properties of *Gal*lysin 2 were not determined (4). Anticancer activity of cationic antimicrobial peptides based on the membranolytic as well as non-membranolytic mode of action was described (25). For example, it was demonstrated that insect antimicrobial peptides, cecropins, exhibited activity against ovarian carcinoma, breast carcinoma and leukaemia cells (5, 6, 22, 27, 29). The results presented in our study suggested that the hemolymph as well as methanolic extracts of *G. mellonella* hemolymph contain compounds inducing apoptosis and/or necrosis of the human brain glioblastoma multiforme cells in the concentration causing 2–3 times increase in the level of the dead cells in the used conditions. Identification of the pro-apoptotic and pro-necrotic factors in *G. mellonella* hemolymph requires further study.

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