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SENSITIZATION OF HUMAN MALIGNANT LYMPHOID CELLS TO ETOPOSIDE BY FUCOIDAN, A BROWN SEAWEED POLYSACCHARIDE

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The search for the substances sensitizing cancer cells to apoptosis induction by chemotherapeutic agents is a task of high importance in the modern strategy of anticancer therapy. *The aim* of the study was to investigate the apoptogenic and apoptosis-modulating activities of fucoidan (sulfated polysaccharide) isolated from far-eastern brown seaweeds *Fucus evanescens* in two human malignant lymphoid cell lines, MT-4 and Namalwa. *Methods:* Apoptosis was assessed morphologically and quantified by flow cytometry analysis of cells stained with propidium iodide. Caspase-3 activation was assayed by flow cytometry with the aid of labeled monoclonal antibodies. *Results:* The fucoidan at 500 µg/ml was not cytotoxic in MT-4 or Namalwa cells even in the setting of long-term presence in culture medium up to 14 days. Nevertheless, pretreatment of MT-4 but not Namalwa cells with fucoidan followed by the exposure to DNA topoisomerase II inhibitor etoposide led to about two-fold increase in the relative apoptotic index as compared with etoposide alone. Apoptosis enhancement of MT-4 cells by fucoidan was not accompanied by further increase in the number of the cells with active form of caspase-3. *Conclusion:* The present findings demonstrate for the first time that fucoidan enhances etoposide induced caspase-dependent cell death pathway in MT-4 but not Namalwa cell line. The mechanisms of such enhancement do not seem to be related directly to caspase-3 activation.

Key words: **fucoidan, brown seaweed, apoptosis, human malignant lymphoid cells, etoposide, caspase-3.**

Etoposide is a widely prescribed and potent anticancer drug belonging to DNA topoisomerase II inhibitors, which stabilize enzyme-DNA cleavage complexes forming nonrepairable protein-linked DNA double strand breaks [1, 2]. The cells injured in such a way are finally eliminated by apoptosis.

Although the most efficacious chemotherapeutic regimens for utilizing DNA topoisomerase II inhibitors were developed over the past several years, their usefulness has been limited by the frequent development of drug resistance. Chemosensitization consisting in using one drug or agent to render cancer cells more susceptible to a second agent represents the novel strategy to enhance the effects of cytotoxic therapeutics [3].

During the past decade, the search of novel substances with considerable potential for chemosensitization was advantageous in revealing the compounds of natural origin possessing the ability of enhancing the cytotoxic activity of chemotherapeutics. For example, deguelin, a plant-derived substance, markedly enhances chemosensitivity of human leukaemia cells to etoposide or cytarabine [4]. Recently, the polysaccharides and peptides, isolated from seaweeds have become a matter of great interest for cancer therapy. The mechanisms of their anticancer activity are related to their ability to suppress the growth of cancer cells (cytotoxic or cytostatic effects), to enhance the immune responses, and to inhibit tumor angiogenesis [5–8]. Several marine algal polysaccharides, fucoidans in particular, have been found to induce apoptosis in cancer cells [9–11]. Nevertheless, the question whether these substances are able to modulate the apoptosis induced by chemotherapeutic agents has been yet unresolved. Combining fucoidan with etoposide may be a novel strategy that has the potential for improving the antineoplastic activity of etoposide. This study was designed to test this hypothesis.

MATERIALS AND METHODS

Materials and reagents. Far-eastern brown seaweeds *F. evanescens*, which have been collected in Okhotsk Sea in August 2002, were used in this study. Etoposide was purchased from Brystol-Myers Squibb SpA (Italy).

Extraction and purification of fucoidan. Fucoidan from *F. evanescens* was prepared as described in [12]. After removal of small shells and mechanical impurities, fresh seaweed *F. evanescens* (25 kg) was extracted with ethanol at a 1:1 ratio at temperature of 50 °C for 24 h. The extract was separated and ethanol was evaporated to obtain a concentrate of biologically active low-molecular substances. The processed seaweeds were dried and extracted with 0.1 M solution of hydrochloric acid (pH 2.0–2.5) for 12 h at 20–25 °C. The extract was concentrated up to 20% of initial volume by ultrafiltration, neutralized to pH 6.0 and evaporated up to 1 L. The concentrate

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was further neutralized and precipitated with two volumes of ethanol. The precipitate was washed out with 96% ethanol and dried. Molecular weigth of fucoidan from *F. evanescens* was 20–50 kDa; with the following monosaccharide content (%): Fuc — 74, Xyl — 6, Man — 1, Glc — 4, Gal — 11; Fuc:SO_4^{2-} mol/mol — 1:(0,7–1). For cell culture experiments, the aqueous stock solution of fucoidan was prepared at 5 mg/ml and sterilized in boiling water bath. The solution was stored at 4 °C prior to the use.

Cell culture and morphology. Human T-acute leukemia cell line MT4 and B-cell lymphoma line Namalwa were obtained from the National Collection of Cell Lines at the Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). The cells were grown in suspension in RPMI-1640 culture medium (Sigma Chem. Co., USA), supplemented with 10% fetal bovine serum (Sigma Chem. Co.) in 5% CO_2 — 95% air atmosphere at 37 °C and 100% humidity. Cells were subcultured once in 3 days by dilution with the fresh medium. Cell viability was evaluated in hemocytometer after staining with 0.1% Trypan blue. For studying cell morphology, cells were cytospined and stained by May—Grunwald—Giemsa technique.

Detection of apoptotic cells. Apoptotic cells with hypodiploid DNA content were detected as previously reported [13]. In brief, cells in exponential growth phase were incubated with 500 µg/ml fucoidan for the time indicated followed by 18 h incubation with etoposide at a dose of 4 µg/ml (MT-4 cells) or 40 µg/ml (Namalwa cells). The cells were washed with ice-cold PBS, harvested and stained with 50 µg/ml propidium iodide (PI) in phosphate-buffered saline containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 µg/ml RNase A. The DNA content of cells was analyzed with FACScan flow cytometer (Becton Dickinson, USA). The relative apoptotic index was calculated as follows:

$$\frac{\text{Apoptotic percentage in presence of inducer} - \text{Spontaneous apoptotic percentage}}{100\% - \text{Spontaneous apoptotic percentage}} \times 100\%.$$

Caspase-3 activation assay. The percentage of cells expressing active form of caspase-3 was assessed by flow cytometry with the use of a FITC-conjugated monoclonal active caspase-3 antibody kit (Becton Dickinson Biosciences, USA). MT-4 or Namalwa cells were pretreated with fucoidan and incubated with or without etoposide as indicated above. The cells were collected and rinsed with ice-cold PBS. Then these cells were permeabilized and immunostained according to the procedure recommended by the manufacturer. Data were analyzed by using CellQuest software (BD Biosciences, USA).

Statistical analysis. The Student *t* test was used to analyze the data. *P* value of < 0.05 was considered statistically significant.

RESULTS

The incubation of both MT-4 and Namalwa cells in the presence of fucoidan at the doses up to 500 µg/ml did not affect cell growth kinetics (Fig. 1).

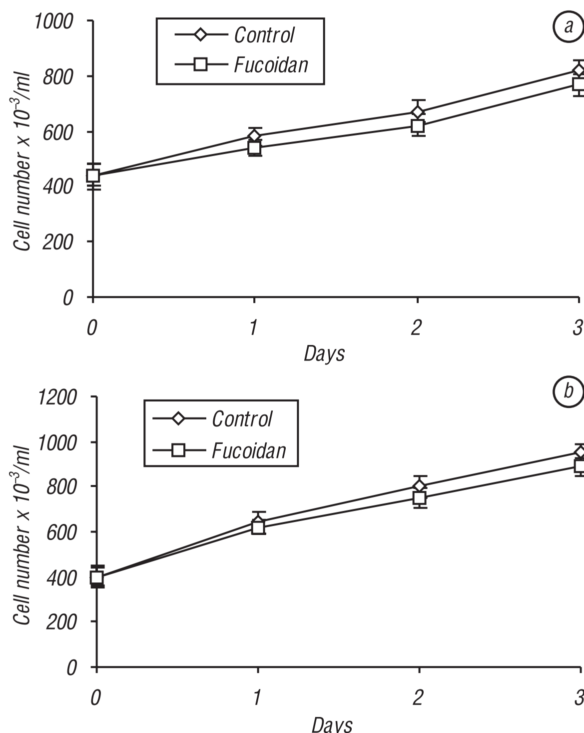

Fig. 1. Effect of fucoidan on growth of MT-4 (*a*) and Namalwa (*b*) cells. Fucoidan was added at 500 µg/ml. Each experiment was performed in triplicate and mean ± S.D. is indicated.

At this maximal dose being assayed, fucoidan did not increase the percentage of apoptotic cells in both cell lines upon 18-hour treatment. Moreover, no apoptotic effect was evident when the cells were incubated in the presence of fucoidan for 14 days with fresh fucoidan solution being added each time the cells were subcultured (Fig. 2, *a*). Upon such a long-term treatment with fucoidan, cell morphology remained unaltered (data not shown).

Pre-incubation with 500 µg/ml fucoidan for 18 h followed by apoptosis induction with etoposide has resulted in about twice as much increase in the percentage of hypodiploid MT-4 cells as compared to the cells treated with etoposide alone. The sensitization effect of the same magnitude was also evident in MT-4 cells incubated with 500 µg/ml fucoidan for 14 days followed by apoptosis induction with etoposide (see Fig. 2, *a*). When Namalwa cells were assayed in the similar experimental setting, the percentage of hypodipoid cells upon etoposide treatment of control culture did not differ significantly from that in the culture pre-incubated with fucoidan (Fig. 2, *b*). No effects of sensitization in Namalwa cells could be detectable upon long-term pretreatment with fucoidan.

Because caspase activation is known to be involved in etoposide-induced apoptosis [14], we attempted to evaluate the percentage of MT-4 cells with the active form of caspase-3 upon their treatment with etoposide alone or etoposide treatment following pre-incubation with fucoidan. As illustrated in Fig. 3, the increase in apoptotic cell percentage in MT-4 cells treated with etoposide after their preincubation with fucoidan was not accompanied by further increase in caspase-3-

positive cells as compared with the cells treated with etoposide only.

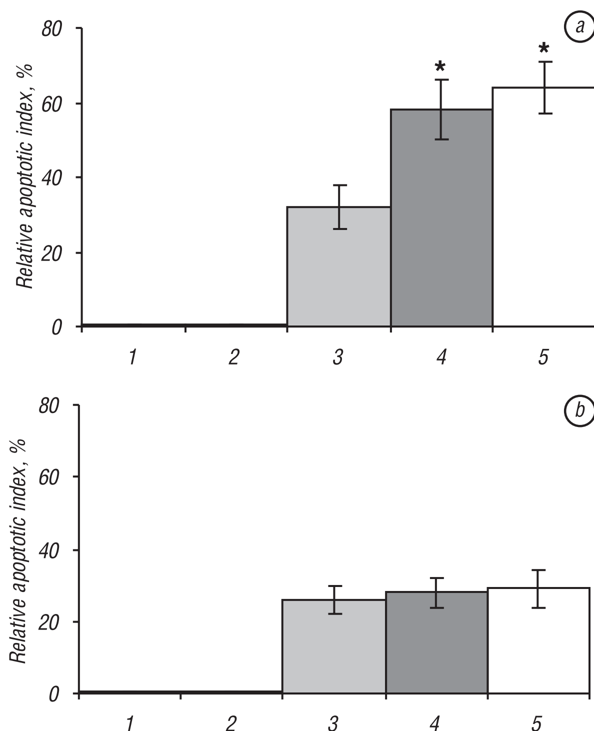

Fig. 2. Relative apoptotic index in MT-4 (*a*) or Namalwa (*b*) cells treated with fucoidan (1 — 500 μg/ml 18 h, 2 — 500 μg/ml 14 days), etoposide (3), fucoidan 18 h + etoposide (4), fucoidan 14 days + etoposide (5). The concentration of etoposide was 4 μg/ml in *a* and 40 μg/ml in *b*. Each experiment was performed in triplicate and mean ± S.D. is indicated
$^*p < 0.01$ vs etoposide alone.

DISCUSSION

Fucoidans are major sulfated polysaccharides of the brown seaweed possessing the wide spectrum of biological activities. There have been many reports on anti-tumor effects of fucoidan [6, 7, 15–17]. Recently, it has been demonstrated that fucoidan may induce apoptosis of cancer cell acting via caspase-3 and -7 activation-dependent pathway and down-regulation of ERK pathways (decreased phosphorylation of ERK and GSK, but not p38 or Akt) [10, 11]. Nevertheless, in our study fucoidan from far-eastern brown seaweeds *F. evanescens* did not possess apoptogenic activity in two lines of human lymphoid malignant cells even upon the prolonged treatment at a concentration of 500 μg/ml. Our data are in line with the results presented recently by Teruya et al. [11] who demonstrated that the native non-modified fucoidan from *Cladosiphon okamuranus* did not decrease cell viability of U937 cells.

Since fucoidan assayed in our study was not capable of inducing apoptosis, we have attempted to investigate whether or not fucoidan could enhance the sensitivity to etoposide of two lines of human lymphoid malignant cells. Earlier we have shown that MT-4 and Namalwa cells are characterized by different intrinsic susceptibility to apoptosis induction by etoposide [13]. Therefore, etoposide dose in our experiments in Namalwa cells was ten-fold as large as that used for apoptosis induction in MT-4 cells providing for about the same apoptotic percentage in both cell lines assayed. In MT-4 cells, fucoidan was shown to be effective as sensitizer towards the apoptogenic effects of etoposide providing for two-fold increase in the apoptotic cell percentage. The sensitization activity of fucoidan was assessed in cells pretreated for 18 h or 14 days. The trend of increment of apoptotic cell percentage with the increasing pre-incubation time was demonstrated although the difference was not significant. In contrast, pretreatment with fucoidan did not affect the susceptibility of Namalwa cells to etoposide.

Caspases are known to be crucial mediators of apoptosis (see review [18] for details). To better understand the probable mechanisms of the apoptosis enhancement by fucoidan, in particular the mechanisms related to caspase-dependent pathway of apoptosis induction in MT-4 cells, we tried to assess the involvement of caspase-3 in sensitization effect of fucoidan in etoposide-induced apoptosis. While etoposide treatment of MT-4 cells resulted in the pronounced caspase-3 activation, the apoptosis enhancement by fucoidan was not accompanied by further increase in the content of active form of caspase-3. Several explanations of this fact could be put forward. The optimal apoptosis induction in MT-4 cells, especially in case of etoposide dose below LD_{50} may require

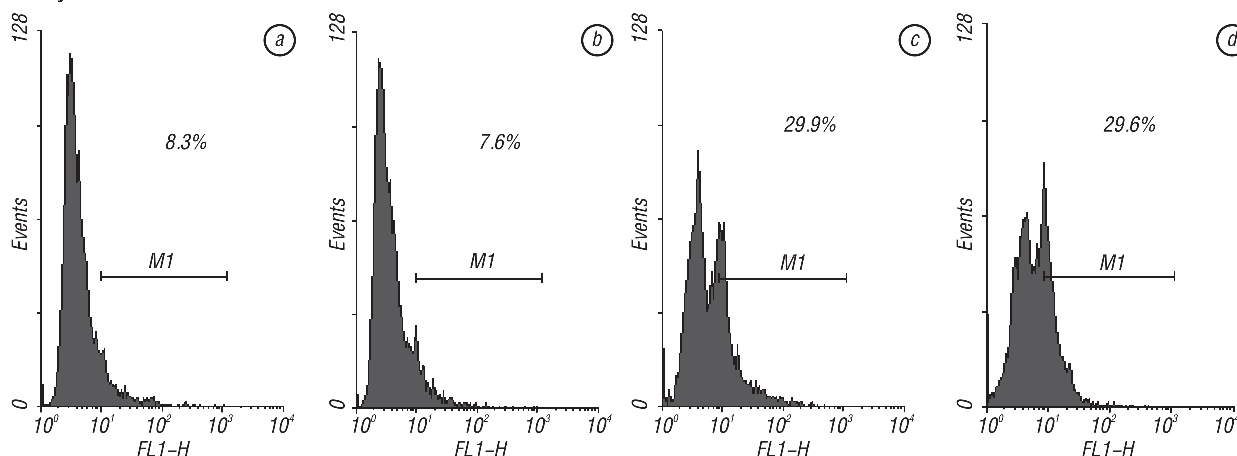

Fig. 3. Active form of caspase-3 in MT-4 cells. *a* — control (without treatment); *b* — fucoidan for 18 h; *c* — etoposide 4 μg/ml; *d* — fucoidan 18 h + etoposide 4 μg/ml. The figures show representative staining profiles for 10,000 cells per experiment. M1 is the cell population defined as caspase-3-positive

some specified level of caspase-3 activation (about 30% according to our data), while further increment in caspase-3 activation may be redundant. Moreover, other proteases are certainly involved in the realization of etoposide-induced apoptosis. The elucidation of the apoptosis-related intracellular targets of fucoidan will be addressed in future studies.

In summary, we demonstrated, to our knowledge for the first time, that fucoidan significantly sensitized human leukemia MT-4 cells to death induced by DNA topoisomerase II inhibitor etoposide. This activity of fucoidan does not seem to be related to caspase-3 activation-dependent pathway. Since in Namalwa cells the sensitization effects of fucoidan were not evident, tumor cell of different origin should be investigated with regard to the cooperative role of fucoidan with topoisomerase II inhibitors.

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ПОВЫШЕНИЕ ЧУВСТВИТЕЛЬНОСТИ ЗЛОКАЧЕСТВЕННЫХ ЛИМФОИДНЫХ КЛЕТОК ЧЕЛОВЕКА К ЭТОПОЗИДУ ПРИ ДЕЙСТВИИ ФУКОИДАНА ПОЛИСАХАРИДА БУРЫХ ВОДОРОСЛЕЙ

Одной из важных задач современной стратегии противоопухолевой терапии является поиск веществ, повышающих чувствительность опухолевых клеток к индукции апоптоза под действием химиопрепаратов. *Цель:* изучение апоптогенной и апоптозмодулирующей активности фукоидана — сульфатированного полисахарида, выделенного из дальневосточной бурой водоросли *Fucus evanescens*, на двух линиях злокачественных лимфоидных клеток человека МТ-4 и Namalwa. *Методы:* апоптоз выявляли морфологически и количественно проточной цитометрией клеток, окрашенных йодистым пропидием. Активацию каспазы-3 изучали методом проточной цитометрии клеток после реакции с конъюгированными моноклональными антителами. *Результаты*: фукоидан в дозе 500 мкг/мл не проявлял токсичности в клетках МТ-4 или Namalwa даже при длительном присутствии препарата в культурах до 14 сут. Предварительная инкубация клеток МТ-4 с фукоиданом в указанной дозе приводила к двукратному повышению относительного апоптотического индекса при действии ингибитора ДНК топоизомеразы II этопозида, причем такого эффекта в клетках Namalwa не отмечено. Повышение апоптотического индекса в клетках МТ-4 под влиянием фукоидана при индукции апоптоза этопозидом не сопровождалось приростом процентного содержания клеток с активной формой каспазы-3, в сравнении с таковым при действии одного лишь индуктора апоптоза этопозида. *Выводы:* впервые продемонстрирована способность фукоидана усиливать этопозидиндуцированный каспазозависимый апоптоз в клетках МТ-4. Подобный эффект отсутствовал в клетках Namalwa. Механизм повышения чувствительности злокачественных лимфоидных клеток человека к этопозиду при действии фукоидана, по-видимому, напрямую не связан с активацией каспазы-3.

Ключевые слова: **фукоидан, бурые водоросли, апоптоз, линии злокачественных лимфоидных клеток человека, этопозид, каспаза-3.**

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