EFFECTS OF TGF–β1, FLUOROURACIL AND CYTOTOXIC LECTINS ON HT–29 AND SW–480 HUMAN COLON CANCER CELLS

E.V. Preobrazhenska, R.S. Stoika

Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv 79005, Ukraine

ВЛИЯНИЕ ТФР–β1, ФЛУОРОУРАЦИЛА И ЦИТОТОКСИЧЕСКИХ ЛЕКТИНОВ НА КЛЕТКИ ОПУХОЛИ ПРЯМОЙ КИШКИ ЧЕЛОВЕКА ЛИНИЙ НТ–29 И SW–480

Е.В. Преображенская, Р.С. Стойка

Институт биологии клетки Национальной Академии наук Украины, Львов, 79005, Украина

Human colon cancer cells of HT-29 line and of SW-480 line (defective in Smad 4 protein function necessary for transforming growth factor β1 (TGF-β1) signalling) were subjected to the action of TGF-β1, anti-cancer drug fluorouracil and some cytotoxic plant lectins. It was found that SW-480 cells were more sensitive than HT-29 cells to growth-inhibiting and cell-killing effects of the cytotoxic mistletoe agglutinins 1 and 2, while the difference in the cytotoxic action of wheat germ agglutinin was not so distinct. TGF-β1 (10 ng/ml) did not affect significantly ^{3}H-thymidine incorporation into DNA of both studied cell lines. These cell lines were similarly inhibited by fluorouracil. The cytotoxic actions of the lectins and fluorouracil were accompanied by strong DNA fragmentation in both cancer cell lines. Potential mechanisms responsible for the appearance of different sensitivity to the cytotoxic lectins in the studied cell lines are discussed.

***Key Words*: human colon cancer cells, HT-29 and SW-480 lines, TGF-β1, plant cytotoxic lectins, fluorouracil, cell growth, apoptosis.**

Исследованы клетки опухоли прямой кишки человека линии НТ-29 и линии SW-480, дефицитной по функции белка Smad 4, необходимого для осуществления сигнальной роли трансформирующим фактором роста β1 (ТФР-β1). Клетки подвергали действию ТФР-β1, противоопухолевого препарата флуороурацила и некоторых цитотоксических растительных лектинов. Установлено, что клетки линии SW-480 значительно более чувствительны, чем клетки линии НТ-29, к рост-ингибирующему и цитотоксическому действию агглютининов 1 и 2 из омелы, тогда как разница в цитотоксическом действии на эти клетки агглютинина из проростков пшеницы менее выражена. ТФР-β1 (10 нг/мл) существенно не влияет на включение ^{3}Н-тимидина в ДНК этих клеточных линий. Флуороурацил оказывает подобный по силе рост-ингибирующий эффект на клетки обеих линий. Действие лектинов и флуороурацила сопровождалось существенной фрагментацией ДНК в исследуемых клетках. Рассмотрены механизмы, которые могут быть ответственны за различия в чувствительности исследуемых линий опухолевых клеток к действию цитотоксических лектинов.

***Ключевые слова*: рак прямой кишки человека, линии НТ-29 и SW-480, ТФР-β1, цитотоксические лектины, флуороурацил, рост клеток, апоптоз.**

Transforming growth factor–β (TGF–β) belongs to a big cytokine family whose members are involved in the regulation of cell proliferation, differentiation, motility and apoptosis [1, 2]. Although TGF–β is one of the most potent inhibitors of normal cell growth, many malignancies of epithelial and hematopoietic origin are resistant to TGF–β action, suggesting that the development of resistance to TGF–β plays an important role in tumorigenesis [3, 4]. Loss of cell sensitivity to TGF–β action was found to be associated with mutations in its specific receptors or deregulation of its intracellular signalling pathway involving Smad proteins [5, 6].

Inactivating mutations in Smad 4 (DPC4) are the most important of the known post–receptor defects in TGF–β pathway in colon cancer cells. Following the initial identification of Smad 4 mutations in half of all pancreatic carcinomas [7], Smad 4 mutations were also reported in colon cancer [8] and other gastrointestinal cancers [9].

Recently it was shown that the escape from TGF–β–triggered inhibition of proliferation and induction of apoptosis in murine L1210 leukemia cells correlated with cell resistance to anti–cancer drug cisplatin [10]. Various anti–cancer drugs and cytotoxic plant lectins were shown to induce an increase in TGF–β1 production in murine leukemia L1210 cells and in human lung adenocarcinoma A–549 cells [11]. Those data suggest TGF–β1 involvement in mediating apoptosis induced by some cell stressing agents.

Taking into account the results mentioned above we studied human colon cancer cells of HT–29 line and of SW–480 line which is defective in Smad 4 protein functioning. The main goal of our study was to check if the loss of a central component in TGF–β signalling pathway could affect the sensitivity of these cancer cells to the action of TGF–β1, anti–cancer drug fluorouracil and some cytotoxic plant lectins.

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Correspondence: E–mail: stoika@biochem.lviv.ua

Abbreviations used: TGF–β1 – transforming growth factor β1; VAA–1 — mistletoe agglutinin 1; VAA–2 — mistletoe agglutinin 2; WGA — wheat germ agglutinin.

MATERIALS AND METHODS

Plant lectins used in this study were: mistletoe (*Viscum album L.*) agglutinin 1 and 2 (VAA–1 and VAA–2), and wheat (*Triticum vulgaris L.*) germ agglutinin (WGA). The lectins were isolated and purified in our laboratory by Dr. M. Lutsik using earlier developed methods [12].

TGF–β1 was purchased from R & D Systems Inc. (Minneapolis, USA) and fluorouracil was bought in the local pharmacy.

Human colon cancer cells of HT–29 and SW–480 lines were obtained from cell culture collection at Ludwig Institute for Cancer Research (Uppsala, Sweden). The cells were cultured in DME medium (Sigma, USA), supplemented with 10% heat–inactivated fetal calf serum (FCS, Sangva, Lviv, Ukraine) and 50 μg/ml gentamycin (Sigma, USA).

Sub–confluent cells were incubated for 24–48 h in the presence of TGF–β1, fluorouracil or cytotoxic lectins. After the completion of the incubation period the number of alive and dead cells was counted in hemocytometric camera after trypan blue staining of dead cells.

^{3}H–thymidine incorporation into cellular DNA was studied after cell seeding in 24–well plates. The studied agents were added to subconfluent cells which were cultured in their presence for 24 h. During the last 5 h of incubation 2 μCi of ^{3}H–thymidine (50 Ci/mmol) in 20 μl FCS–free culture medium was added to each well. The cell monolayer was washed 2 times with ice–cold phosphate buffered saline, 2 times with 5% TCA and the amount of radioactivity was quantified in TCA–insoluble materials with β–scintillation counter (LKB, Sweden).

DNA fragmentation was studied after DNA preparation and electrophoresis performed as described [13]. Briefly, $2 \cdot 10^{6}$ cultured cells were pelleted and then resuspended in 50 μl lysis buffer containing 50 mM Tris–HCl (pH 7.5), 20 mM EDTA, 1% NP–40. SDS (final concentration 1%, Serva, Germany) and RNase A (final concentration 1 mg/ml, Sigma, USA) were added to each sample, which was then incubated for 1 h at 37 °C. After that proteinase K (final concentration 1 mg/ml, Sigma, USA) was added and samples were incubated for 1 h at 37 °C. DNA was pelleted by adding 10 M ammonium acetate ((1/2 of sample volume) and 2 volumes of ice–cold isopropanol at –20 °C overnight. Samples were centrifuged for 30 min at 10,000 g, pellets were air–dried, dissolved in TE–buffer (10 μl/10^{6} cells) and loaded into the wells of 1% agarose gel. Electrophoresis was carried out in TAE–buffer (pH 8.0) in the presence of ethydium bromide and the gel was examined in transilluminator (LKB, Sweden) under UV light and photographed.

For statistical analysis each experiment was performed in triplicate and repeated three times. Significance of the difference in a typical experiment was assessed by Student's *t*–test with the level of significance set at 0.05.

RESULTS

It was shown in many studies that TGF–β1 inhibits growth of the mammalian epithelial tissue cells [3]. However, we did not reveal the effect of this cytokine (10 ng/ml) on ^{3}H–thymidine incorporation neither in HT–29 line possessing intact TGF–β1 signalling pathway, nor in SW–480 line possessing an impaired TGF–β1 signalling due to a defective Smad 4 functioning (Fig. 1). The mistletoe lectin VAA–1 decreased ^{3}H–thymidine incorporation in both studied cell lines, although such action was more pronounced in the cells of SW–480 line than in the cells of HT–29 line (Fig. 2). That is why different VAA–1 concentrations were used in the experiments: 20 ng/ml for SW–480 line and 200 ng/ml for HT–29 line. TGF–β1 (10 ng/ml) did not affect the inhibiting action of the mistletoe lectin.

The above noted difference between colon cancer cells of HT–29 and SW–480 line in their growth response to the mistletoe agglutinin was further confirmed when its dose dependent action on total cell number and dead cell number was studied. Both isoforms of the mistletoe agglutinin — VAA–1 and VAA–2 — markedly inhibited growth of SW–480 cells, while their action on HT–29 cells was less pronounced (see Fig. 2). That difference corresponded to the effect of those lectins on the amount of dead colon cancer cells, which

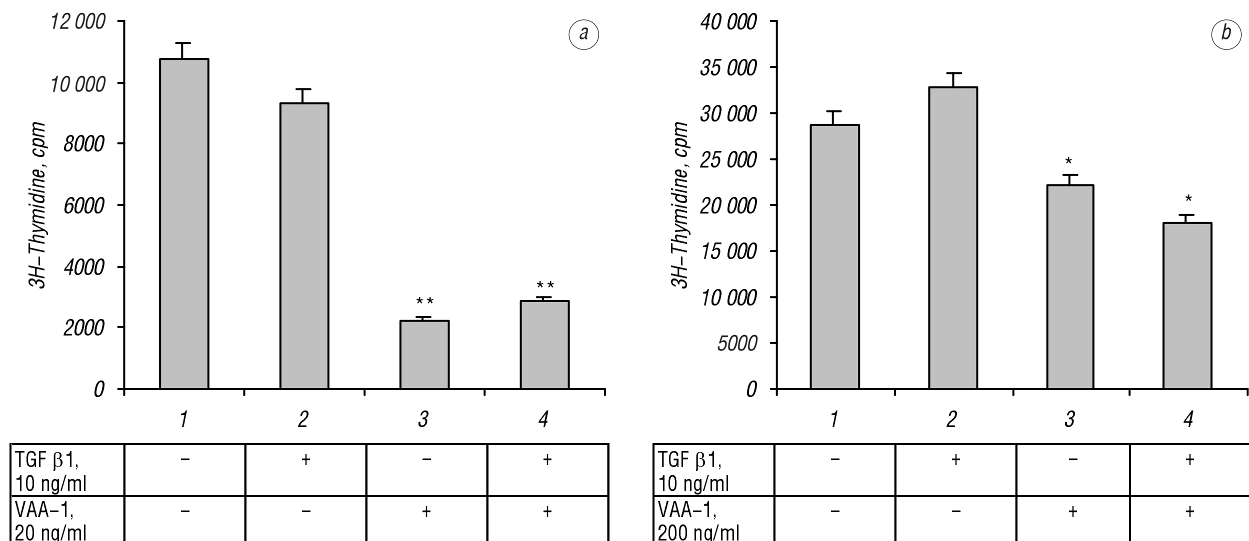

Fig. 1. Effect of TGFβ1 and VAA–1 on ^{3}H–thymidine incorporation into DNA of growing human colon cancer cells of SW–480 (*a*) and HT–29 (*b*) lines.
* $P < 0.05$; ** $P < 0.01$

was much higher in the case of SW–480 cells in comparison with HT–29 cells. It should be noted that such difference in colon carcinoma cells sensitivity to the inhibiting action of the lectin was specific for the mistletoe lectin as it was detected that WGA inhibited growth of HT–29 cells to a higher extent than it inhibited growth of SW–480 cells (see Fig. 2). Dose dependence patterns of WGA cytotoxic effects were similar for both colon cancer cell lines.

Dose dependence of the anti–cancer drug fluorouracil effect on the total cell number and the number of dead cells in HT–29 and SW–480 colon cancer cell lines did not differ significantly (Fig. 3).

In order to know the mechanisms of the killing effect of various stressing agents acting on human colon cancer cells of HT–29 and SW–480 lines, DNA fragmentation was studied in those cells. It was found that mistletoe agglutinin isoforms VAA–1 and VAA–2, as well as the WGA, induced apoptosis in both lines of the colon cancer cells (Fig. 4). The anti–cancer drug fluorouracil acted in a similar way. It should be noted that the epithelial tissue cells, usually, do not show a distinct DNA «ladder» which can be observed when the mesenchymal tissue cells were studied for the appearance of apoptotic DNA fragmentation [14]. The reason for that could be either the expression of various types of DNA fragmentation, or the simultaneous expression of apoptosis and necrosis in the epithelial tissue cells under the effect of stressing agent action.

DISCUSSION

Human colon cancer cells of SW–480 line have been extensively used as an experimental model for *in vitro* studies of the role of TGF–$\beta 1$ regulatory system in tumorigenesis. These cells contain some genetic alterations characteristic for colon cancer, namely the activating mutations in Ki–*ras* oncogene, both a complete deletion and a stop codon mutation in the APC gene

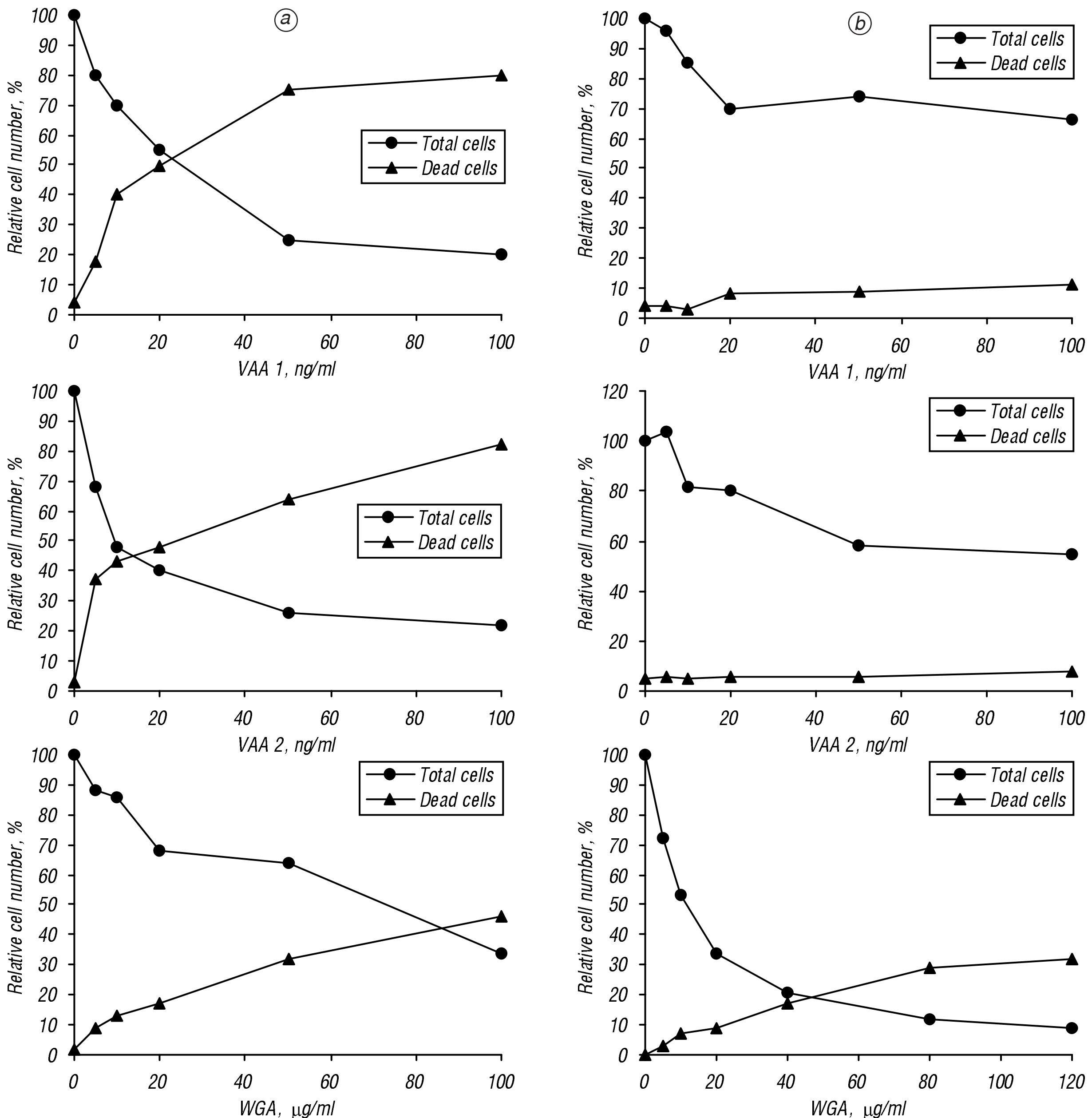

Fig. 2. Dose dependence of the effect of cytotoxic plant lectins on growing human colon cancer cells of SW–480 (*a*) and HT–29 (*b*) lines

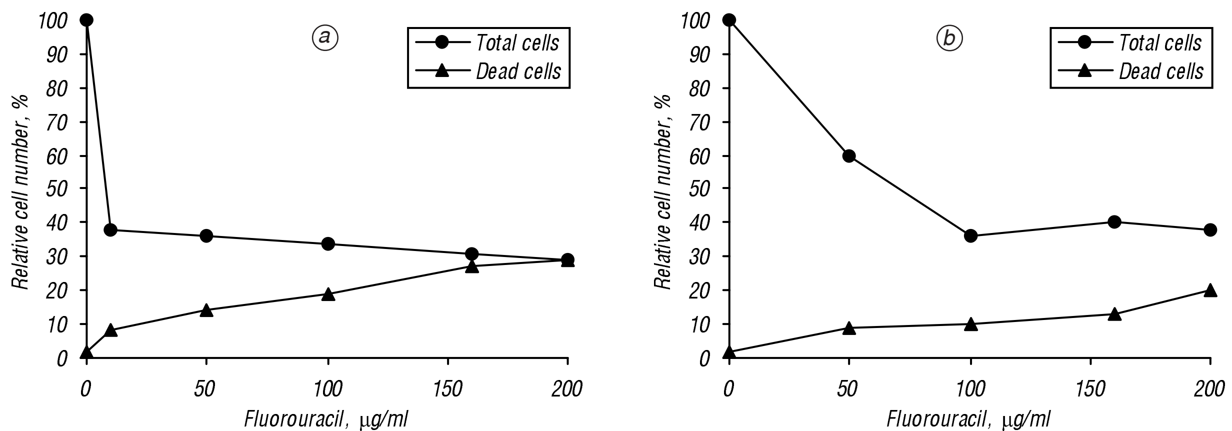

Fig. 3. Dose dependence of the effect of fluorouracil on growing human colon cancer cells of SW–480 (a) and HT–29 (b) lines

region, and inactivating mutations in both p53 alleles [15]. Additionally, these cells have lost one copy of chromosome 18 where the Smad 4 locus is found (18q21). One wild type copy of Smad 4 is retained, however it is expressed in a very low level [16].

HT–29 is another human colon cancer cell line widely used in the experimental oncology. This cell line retains many biochemical and physiological features (intact Smad 4 alleles) characteristic for normal colorectal epithelial cells [17]. Usually, HT–29 cells are used as a control for comparison with Smad 4 defective colon carcinoma SW–480 cells.

Smad 4 plays a central role in TGF–β1 signalling by serving as a common partner for the other Smad proteins [5, 6]. It was shown that TGF–β1 binding brings together two types of transmembrane serine kinases which are type I and type II TGF–β receptors. In this complex, the type II receptor phosphorylates and activates the type I receptor, which, in turn, phosphorylates Smad 2 or the highly related protein Smad 3. Upon phosphorylation, these Smad proteins associate with Smad 4 and move to cell nucleus where they assemble transcription complexes that activate specific sets of genes. Thus, Smad 4 is a shared key component in TGF–β1 signalling pathway.

We did not reveal TGF–β1's effect on ^{3}H–thymidine incorporation into DNA of the studied colon cancer cells. Such result could be understood in the case of SW–480 cells lacking Smad 4 protein function which is necessary for TGF–β1 signalling. The lack of TGF–β1 effect in the case of HT–29 cells could be explained by the presence of 10 % FCS which may mask TGF–β1's growth inhibiting action. However, other reasons, such as the use of HT–29 colon cancer cells from a different source cannot be excluded.

5–Fluorouracil is an anti–cancer drug widely used for treatment of the patients with tumors of the gastro–intestinal tract, including the colon cancers [18]. It competes with uracil and thus blocks thymidine synthesis from uridine. Being incorporated into DNA and RNA it inhibits DNA and RNA biosynthesis. We found that fluorouracil inhibits ^{3}H–thymidine incorporation into the DNA of both HT–29 and SW–480 colon cancer cells. Earlier [11] we found that some anti–tumor drugs induced tumor cells to TGF–β1 production. Taking into account growth–inhibiting and apoptosis–inducing effects of TGF–β1 in those tumor cells it was suggested that this cytokine may partly mediate negative effect of anti–tumor drugs. Data presented in this study do not support such possibility for the colon cancer cells of HT–29 and SW–480 lines, although the induction of TGF–β1 production by fluorouracil cannot be excluded (experiments are in progress).

The cytotoxic plant lectins were proposed to be used as antitumor drugs long ago [19]. Mistletoe (*Viscum album L.*) agglutinins were also tested for their antitumor activity [20, 21]. Many cytotoxic lectins consist of two types of the polypeptide chains: one is possessing affinity to specific carbohydrate moieties, while another is possessing enzymatic activity capable of blocking protein synthesis on the ribosomes [22].

We found that colon cancer cells of SW–480 line were more sensitive to the mistletoe agglutinin isoforms VAA–1 and VAA–2 in comparison with the colon cancer cells of HT–29 line. Taking into account that the cells of SW–480 line have lost their sensitivity to growth inhibitor (TGF–β1) [16] those cells may be considered more transformed than the cells of HT–29 line. Thus,

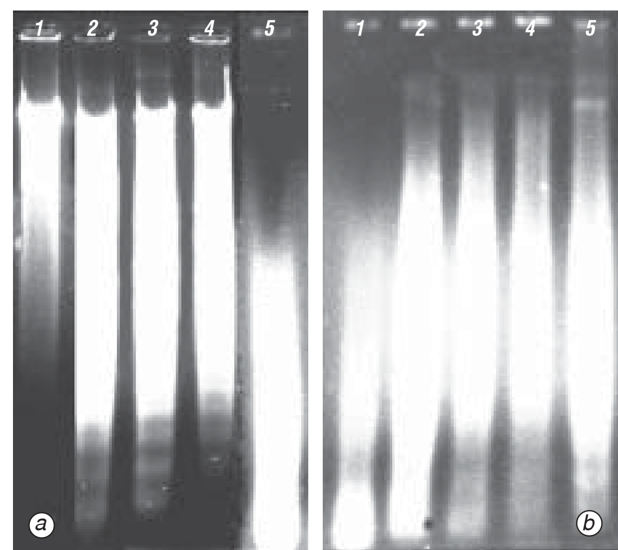

Fig. 4. Induction of DNA fragmentation by cytotoxic plant lectins and fluorouracil in growing human colon cancer cells of SW–480 (*a*) and HT–29 (*b*) lines.
1 – untreated cell; 2 — VAA–1 (20 ng/ml for (*a*) and 200 ng/ml for (*b*)); 3 — VAA–2 (20 ng/ml for (*a*) and 200 ng/ml for (*b*)); 4 – WGA (20 ng/ml); 5 — fluorouracil (50 µg/ml)

more transformed cancer cells are, nevertheless, more susceptible to the cytotoxic action of the mistletoe lectins. The results of DNA fragmentation study suggest that the way of death of the studied colon cancer cells under the action of the cytotoxic lectins is both apoptosis and necrosis.

Earlier we showed that cisplatin– and TGF–β1–resistant murine leukemia cells of L1210 line are more sensitive to the mistletoe agglutinin in comparison with cisplatin– and TGF–β1–sensitive cells of the same line [10]. We also found that TGF–β1–resistant L1210 cells are better stained by specific plant lectins comparing with TGF–β1–sensitive L1210 cells [23]. Besides, more transformed cells of L1210 line were better agglutinated by various plant lectins in comparison with less transformed cells of this line [23]. Those data suggest that the malignant cell transformation may induce the expression of specific plasma membrane markers which can be recognised by specific plant lectins. One of such markers (with molecular weight around 200 kDa) was detected in TGF–β1–resistant murine leukemia L1210 cells [23].

At the moment, we do not possess direct data on the expression of specific plasma membrane marker(s) in the TGF–β1–resistant colon cancer cells of SW–480 line. However, the carbohydrate specificity of such marker can be predicted, as another plant lectin WGA inhibited growth of SW–480 and HT–29 colon cancer cells to a similar extent. Other investigators [24] used a differential ability to bind peanut agglutinin (PNA) and WGA in various human colon cancer cells, particularly in SW–480 and HT–29 lines, as well as the metastatic line SW–620, for the lectin–targeted anti–cancer drug doxorubicine delivery to selected cancer cell lines. The targeting moieties and doxorubicine were conjugated to a water–soluble co–polymer based on N–(2–hydroxyprolyl) methacrylamide acting as a carrier responsible for controlled intracellular release of the targeted drug. FACS analysis showed a strong binding of WGA–FITC to all tested cell lines, while PNA–FITC binding was considerably weaker. While the conjugates with WGA were more cytotoxic, the conjugates with PNA were more specific and their binding was limited to cancer cells and to the sites of inflammation. These results support the idea of site–specific lectinotherapy of malignancies.

In conclusion, the results of our study suggest that specific cytotoxic lectins could be used as an instrument for estimating the level of the malignant transformation in the mammalian cells. It is probable that plasma membrane components of more transformed cells bind specific plant lectins more effectively and, thus, may be more sensitive to the cytotoxic action of those lectins. Besides, it is obvious that in more transformed cancer cells TGF–β1 resistance may appear.

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