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ARTICLE

P-glycoprotein Expression is Induced in Human Pancreatic Cancer Xenografts During Treatment with a Cell Cycle Regulator, Mimosine

Attila ZALATNAI

1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Faculty of Medicine

Application of several cell cycle checkpoint regulators seem to be promising in various experimental models including pancreatic cancer, and they are being evaluated in Phase I-II clinical trials. Among these compounds, mimosine, a plant-derived amino acid has shown an antineoplastic effect on human lung or pancreatic cancer xenografts in addition to cell cycle arrest in the late G1 phase. In the present study, immunosuppressed CBA mice bearing subcutaneously growing human ductal pancreatic adenocarcinomas were treated with 30 mg/kg L-mimosine for 34 days. The treatment resulted in retardation of tumor growth, accompanied by a significantly diminished proliferative activity (22.6% ± 1.7% Ki-67 positivity vs. 29.9% ± 1.1% in controls, mean ± SEM, P < 0.007) and an increased apoptotic rate (14.5 ± 1.1 apoptotic cells/mm² vs. 3.8 ± 0.4/mm² in the controls, P < 0.0001). The immunohistochemical expression of the multidrug resistance gene (MDR1)-encoded P-glycoprotein (p170) was studied. The parental and the untreated tumors did not express p170 protein, but in the mimosine-treated samples 30 to 60% of the carcinoma cells displayed a linear, membrane-bound positivity. The results indicate that P-glycoprotein is inducible by a cell cycle regulator, creating an acquired resistant phenotype.(Pathology Oncology Research Vol 11, No 3, 164–169)

Key words: P-glycoprotein, pancreatic cancer, xenografts, human tumors, mimosine

Introduction

Pancreatic cancer still remains a challenge in oncology because of its rising incidence and the poor survival results. First-line chemotherapeutic agents offer a modest objective response rate for the majority of patients, therefore, continuous search for novel therapeutic modalities is imperative. Nowadays, in the era of targeted treatment strategies, cell cycle regulators have become one of the potential antineoplastic molecules that are extensively studied, and some of them are in Phase I-II clinical trials. Promising preclinical studies have raised the possibility that various checkpoint regulators affecting G1 progression and/or G1-S transition might also be suitable candidates for cancer therapy.[4,24,40]

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Correspondence: Dr. med. habil. Attila ZALATNAI, 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Faculty of Medicine. Üllői út 26., Budapest, H-1085, Hungary. Tel.: +(36-1) 266-1638; 215-6920, fax: +(36-1) 317-1074, E-mail: zalatnai@korb1.sote.hu

For pancreatic cancer, some encouraging experimental data are also available on the synchronizing compounds that lead to cell cycle arrest in specific phases. Treatment of human pancreatic cancer cells with butyrolactone-I resulted in a significantly inhibited RB protein phosphorylation and cyclin A expression, and apoptotic cell death was evidenced by an increased Bax/Bcl-2 ratio in TUNEL assay.[35] Staurosporine, which is a universal protein kinase C inhibitor and suppresses CDK2 activity, has not been tested in this tumor, but its derivate, 7-hydroxystaurosporine (UCN-01) proved to be promising: using pancreatic cancer xenografts it exhibited G1 arrest, significantly decreased the growth of the tumors, induced a marked p21 protein expression and increased the level of apoptosis.[1] Aphidicolin that results in an accumulation of the cells at G1/S transition, induced damage to the DNA in MiaPaCa cells by arresting the replication forks.[30] Histone deacetylase inhibitors (for example FR901228, Depsipeptide) produce mitotic arrest with G2-M accumulation in different cell lines,[27] and Phase I trials have shown some antitumor effects in refractory neoplasms.[26] This compound was also tested using different human pancreatic cancer cell lines,

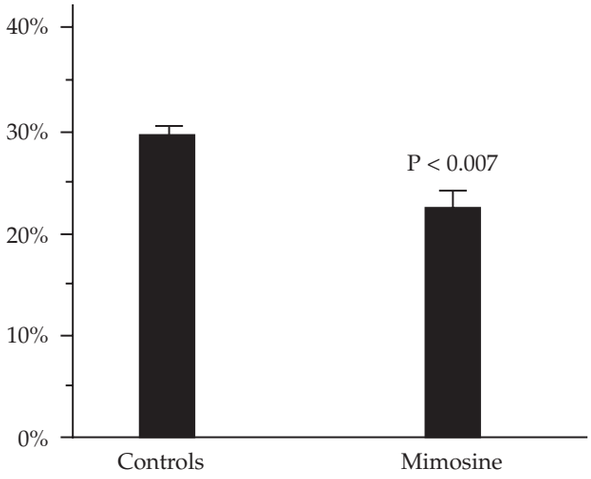

Figure 1. *Proliferative activity in the control and mimosine-treated pancreatic cancer xenografts. Data are expressed as percentage of Ki-67 positive nuclei (mean ± SEM).*

where cell cycle arrest was accompanied by a markedly inhibited proliferation, apoptosis induction, overexpression of $p21^{WAF-1}$, or activation of caspase-3.[28] Experimental data also showed that the selective proteasome inhibitor bortezomib enhanced the antineoplastic activity of taxanes in orthotopic pancreatic cancer xenografts: significantly reduced tumor volume, inhibition of proliferation, p21 accumulation, or increased apoptosis were observed after bortezomib plus docetaxel combination treatment.[21]

In the line of cell cycle regulators, mimosine, a plant-derived, non-protein amino acid produced by *Mimosa* and *Leucocaena* species has long been used in experimental studies. It effectively prevents DNA synthesis by blocking the late G1 phase,[14,34,38] interferes with the synthesis of histone H1 kinase,[10] specifically inhibits cyclin D1 expression,[5] and upregulates p27 protein level.[36] In vivo studies have also demonstrated an antineoplastic effect on human lung cancer xenografts.[6] In earlier studies we provided evidence that mimosine suppressed the growth of subcutaneously transplanted human pancreatic cancer xenografts, and flow cytometric results have shown a significantly elevated sub-G1 fraction indicative of apoptosis.[41] In the present investigation the effect of mimosine treatment on the immunohistochemical expression of P-glycoprotein was studied in the same experimental model.

Chemoresistant phenotype of cancer cells that restricts the success of therapeutic efforts is resulted from complex mechanisms. The major (but not the only) cause of this phenomenon is the intrinsic or acquired overexpression of multidrug resistance gene (MDR1) that encodes the membrane-bound P-glycoprotein (P-gp). The latter acts as an ATP-dependent efflux-pump leading to a decreased drug accumulation inside the tumor cells. Indeed, MDR1 RNA levels were usually found to be elevated in a large number of untreated drug-resistant malignancies including islet cell tumors of the pancreas.[11] One of the therapeutic strategies is to develop P-gp inhibitors of high potency and specificity, and some clinical trials have indeed shown benefits with the use of these agents alone or in combination.[16,32] On the other hand, however, negative MDR1/P-gp status of a given tumor does not necessarily guarantee the success of chemotherapy, because during tumor progression, or due to the effect of treatment itself, the P-glycoprotein may be induced. Such induction of P-gp has been observed in a gastric cancer xenograft system,[20] and in patients with breast cancer or soft tissue sarcomas,[2,8,13] but radiotherapy has also been reported to induce a highly significant P-gp overexpression in oral cancer.[22]

Materials and methods

Xenografts

Establishment of xenograft tumors in immunosuppressed mice was carried out as it was described earlier.[3] Briefly, chunks of freshly resected human pancreatic carcinomas were inoculated s.c. into the back region of inbred, artificially immunosuppressed CBA mice. The animals were kept in the air-conditioned Animal Facility with 55% of relative humidity and at a temperature of 25ºC with 12/12 hours dark/light cycle. Histologically, the tumors were Grade I ductal adenocarcinomas and encoded as PZX-40. For this experiment tumor-bearing animals from the 8th passage were used.

Chemicals

L-mimosine (from Koa hoale seeds) was purchased from SIGMA-Aldrich Ltd. (St. Louis, MO, Cat. no.: M 0253). It was first dissolved in Tris-buffer at pH 8.9, then pH was adjusted to 7.2 with 1 N HCl. Before administration, this solution was freshly diluted by physiologic saline to achieve the required concentration.

Experimental design

The experiment was started when the average diameter of the xenografted tumorous nodules was about 0.5–0.6 cm. After randomization the animals have been divided into 2 groups: untreated controls (n=10) and mimosine-treated mice (n=10). Animals have been treated for 34 days as it was described before.[41] Mimosine was injected subcutaneously at a dose of 30 mg/kg body weight once a day, 6 times a week. The control mice were given 0.9% saline alone. Tumor diameters were measured weekly by microcaliper, and relative volume changes were calculated. At the end of the experiment the animals were sacrificed, and the removed tumors were fixed in buffered formalin and embedded in paraplast.

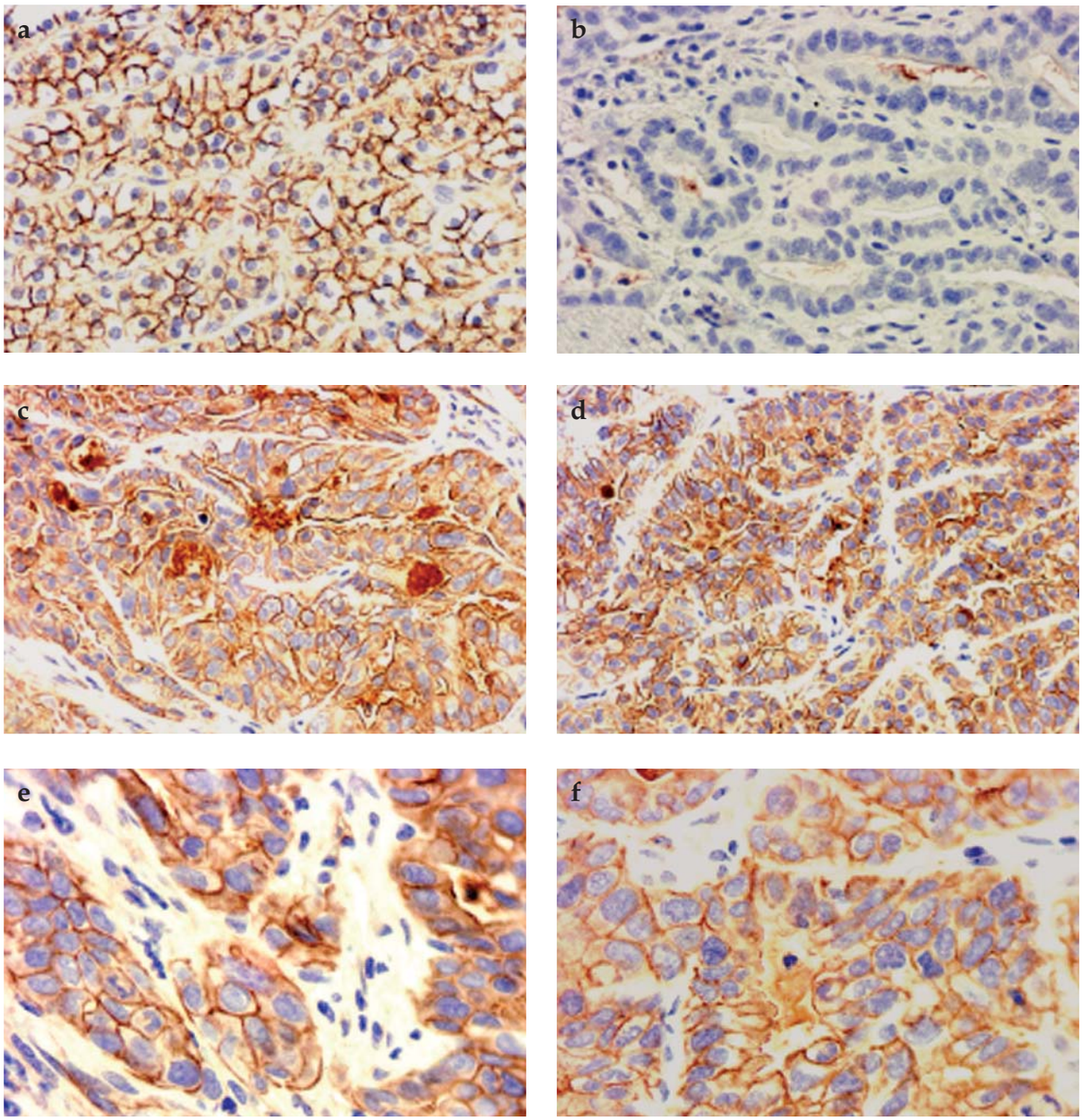

Figure 2. *Immunohistochemical expression of P-glycoprotein.* ***(a)*** *Adrenal cortical cells serving as positive control. Positive reaction is identified as a linear staining in the cell membranes. (x200);* ***(b)*** *PZX-40/8 human pancreatic cancer xenograft without mimosine treatment. No specific positive reaction is seen in the cellular membranes (x 200);* ***(c-f)*** *PZX-40/8 human pancreatic cancer xenografts after a 34-day mimosine treatment. Most of the tumor cells display a linear positive cytoplasmic staining for P-gp* ***(c-d:*** *x200,* ***e-f:*** *x400)*

Evaluation

For identification of the P-glycoprotein/p170 an immunohistochemical method was applied using adrenal gland as a positive control. Immunostaining was performed by using a monoclonal human anti-P-glycoprotein primary antibody (Clone C494, NeoMarkers, Fremont, CA) and streptavidin-biotin peroxidase (LSAB2) system (DAKO, Glostrup, Denmark). DAB was used as a chromogen, and the specimens were counterstained by hematoxylin. The immune reaction was accepted as positive in case of linear staining in the cell membrane, and the pro-

portion of immunoreactive carcinoma cells to the whole tumorous population was assessed.

Proliferation activity was assessed by Ki-67 score (DAKO, Clone Mib-1), and the proportion of the positive nuclei was calculated by using an ImageTool software (UTHSCSA, San Antonio, TX).

The number of mitotic and apoptotic tumor cells have been counted in high-power fields (x40 objective, x10 ocular) in H&E stained sections. Only the areas of tumorous tissue were encountered, necrotic parts have been disregarded. The total area of each microscopic field was 0.19 mm^2, and the final values were expressed as a number of mitoses per mm^2. Determination of the mitotic activity was based on screening of 30 HPFs. Student's t-test was applied for evaluating significance by using a PlotIt 3.02 software (Haslett, MI).

Results

All but one animals survived the experiment. The only spontaneous death occurred in the mimosine group as a result of irradiation-induced progressive atrophy. Mimosine treatment has resulted in a growth delay in the xenografts (188% tumor volume increase in controls vs. 121% in treated animals), and the proliferative activity has significantly diminished. While in the untreated tumors the proportion of Ki-67 positive nuclei was 29.9% ± 1.1%, in the mimosine-treated samples it proved to be 22.6% ± 1.7% ($P < 0.007$) (*Figure 1*). Similarly, there was a highly significant increase in the apoptotic activity: 14.5 ± 1.1 apoptotic cells per square mm in the mimosine group vs. 3.8 ± 0.4/mm^2 in the controls ($P < 0.0001$).

The anti-p170 antibody visualized the membrane-bound form of the P-glycoprotein (*Figure 2a*). No specific immunohistochemical expression was seen in samples from the parental tumor (not shown), or the untreated xenograft (*Figure 2b.*), but the intraluminal mucin gave some aspecific reaction products. In all mimosine-treated tumor samples (n=9), however, p170 expression of variable degree was noted. The carcinoma cells displayed a continuous circular or semi-circular positive reaction at the cytoplasmic membranes, that was present in 30–60% of the tumorous elements (mean: 40%). No cytoplasmic or nuclear stainings were observed. There was no zonal arrangement of the positive cells within the tumors, and the presence or absence of necrotic areas did not influence the positivity either (*Figures. 2c-f*). The stromal cells were uniformly free of immunostaining.

Discussion

The prognosis of pancreatic carcinoma is usually grim, because the tumor is largely resistant to chemotherapy. It seems that this feature is not directly linked to MDR1/P-gp expression, because decreased cytostatic drug accumulation has also been observed when P-glycoprotein was not demonstrable, suggesting an alternative transport mechanism.[16] The MDR1 gene expression is more significantly elevated in pancreatic carcinoma than that is in normal pancreata, and over 70% of ductal adenocarcinomas are highly positive for P-gp,[31] however, the clinical aggressiveness of the tumor does not depend on the presence or absence of P-gp.[31] Moreover, the survival of MDR1-positive and MDR1-negative pancreatic cancer patients who underwent surgery did not differ significantly.[18]

The malignant cells cannot be regarded as stable structures, since they continuously change their phenotype in answer to the environmental influences. Better adapted tumors gain selection advantage. It was reported, for example, that in bcl-2 negative human pancreatic cancer xenografts octreotide (Sandostatin) treatment has induced the immunohistochemical expression of this antiapoptotic protein.[39] Several experimental data demonstrate that an originally MDR1-negative tumor may become resistant after chemotherapeutic treatments, as it was shown in Ehrlich ascites cells,[12,23] rat liver epithelial cells,[9] ovarian cancer cells[23,29] and some other cell lines.[7] The cytostatic drugs induced MDR1 transcripts, functional overexpression of P-gp, resulting in a requirement of larger tumor-inhibiting doses.[12] The acquired resistance has usually been sustained for several weeks,[17] and its induction may be drug-specific. Licht and al. have reported that in a mesothelioma cell line originally expressing less than 1% of P-gp, the MDR-substrate cytostatic drugs (vincristine, vinblastine or doxorubicin) induced the MDR, but exposure to cisplatin or cyclophosphamide resulted in a negligible induction.[17] Sometimes a short (1-hour) exposure is enough to achieve an acquired resistant phenotype in vitro as it was reported in human colon cancer cells.[33] An interesting finding is that the drugs may induce resistance not only to the given chemotherapeutic agents, but concomitant cross-resistance can also be observed.[25,33] In addition, some antibiotics are also able to stimulate overexpression of P-glycoprotein, similarly to doxorubicin.[19] This observation is worth mentioning, because cancer patients are frequently complicated by infectious diseases, and doxycycline might, inadvertently, negatively influence the chemosensitivity of the tumor.

Concerning the cell cycle regulators, experimental data are very limited and derive from in vitro studies. Wartenberg et al. have investigated prostate tumor spheroids, and found that treatment with the promiscuous protein kinase C inhibitor staurosporine resulted in a downregulation of P-gp, mitomycin C or roscovitine had no significant effect, but mimosine upregulated the P-glycoprotein.[37] So far this has been the only experimental result about the connection between mimosine and multidrug resistance, but it clearly shows that cell cycle regulators have multiple targets of attack.

The phenomenon that originally sensitive tumors become resistant to the treatments is well known from the clinical practice, but it is not easy to decide whether it is due to clonal evolution of the neoplasm or it is induced by the cytostatic drugs. Both mechanisms may be taken into account. Here we presented experimental evidence that a cell cycle regulator was able to induce P-glycoprotein overexpression in an originally P-gp-negative pancreatic cancer xenograft, creating an acquired resistant phenotype, although the biological resistance of this tumor remains to be demonstrated. Such effect of mimosine has not been known before.

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