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Potential role of transforming growth factor β1 in drug resistance of tumor cells★✪

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Acquired drug resistance of tumor cells is frequently observed in cancer patients undergoing chemotherapy. We studied murine leukemia L1210 cells sensitive and resistant to the cytotoxic action of cisplatin and showed that cisplatin-resistant leukemia cells were also refractory to TGF β1-dependent growth inhibition and apoptosis. Addressing the question about the mechanisms responsible for the cross-resistance to cisplatin and TGF β1, we found that cisplatin- and TGF β1-resistant L1210 cells possessed a decreased expression of type I TGF β1 receptor, while the expression of type II TGF β1 receptor was not affected. Western blot analysis of Smad proteins 2, 3, 4, 6, and 7, which participate in signal transduction pathway down-stream of the TGF β1 receptors, revealed an increased expression of Smad 6, inhibiting TGF β1 action, only in cisplatin- and TGF β1-resistant L1210 cells. TGF β1 and especially the cytotoxic mistletoe agglutinin increased Smad 6 expression in TGF β1-sensitive but not in TGF β1-resistant L1210 cells. TGF β1-resistant L1210 cells also differed from TGF β1-sensitive cells by the lack of expression of the pro-apoptotic p53 protein and higher

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Abbreviations: Con A, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; L1210/S, parental cisplatin-sensitive L1210 cells; L1210/R, derivative cisplatin-resistant L1210 cells; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RCA-120, *Ricinus communis* agglutinin 120 kDa; TGFβ1, transforming growth factor β1; TβRI and TβRII, type I and type II receptors for TGF β1; VAA-1 and 2, mistletoe (*Viscum album*) agglutinin-1 and 2; WGA, wheat germ agglutinin.

level of expression of the anti-apoptotic Bcl-2 protein. Thus, the described co-expression of tumor cell refractoriness to an anti-cancer drug and to the inhibitory cytokine TGF β1 is accompanied by multiple changes in the TGF β1 signal transduction pathway and in other regulatory systems of the target cells. Besides, we found that various anti-tumor drugs and cytotoxic plant lectins increased the level of TGF β1 expression in both TGF β1-sensitive and -resistant L1210 cells. A hypothesis is proposed that TGF β1 can at least partly mediate the effect of cell-stressing agents and, thus, the development of TGF β1 resistance may be responsible for the appearance of tumor cell refractoriness to the action of some anti-cancer drugs.

Acquired resistance to specific anti-tumor drugs is encountered in about one third of all cancer patients undergoing chemotherapy (Young, 1989). This creates serious clinical problems substantially limiting the efficacy of anti-cancer drugs. Various mechanisms have been proposed to explain the development of drug resistance in tumor cells (for reviews see Shishova & Chekhun, 1998; Waxman, 1990; Krishan *et al.*, 1997; Brown & Woulters, 1999): 1) impaired drug transport inside and from within the cell; 2) increased efficacy of intracellular detoxication mechanisms and DNA repair; 3) increased anti-apoptotic potential of tumor cells. However, none of these mechanisms have been accepted as universal or dominating. Thus, they cannot explain all appearing cases of drug resistance in tumor cells.

An early event in the development of many malignancies is loss of response to growth control, including cell release from TGF β1 -dependent growth inhibition (Pasche, 2001; Souchelnytskyi, 2002). A number of TGF β1 binding receptors have been identified on the cell surface, but only type I (TβRI), type II (TβRII) and type III receptor (beta-glycan and endoglin) functions in the signalling have been described (Heldin *et al.*, 1997; Massague, 1998). TβRI and TβRII were shown to be essential for signal transduction. Defects in TGF β1 signalling were shown to happen due to mutations in genes coding for the type I (Cen *et al.*, 1998) or type II (Derynck *et al.*, 2001) TGF β1 receptors.

It was shown that Smad proteins function in TGF β1 signalling down-stream of its receptors (Heldin *et al.*, 1997; Massague, 1998). Smad proteins are divided in three groups, depending on their role in the signal transduction pathway. Smad 2 and Smad 3 belong to the first group and are directly activated by TGF β1 receptor-dependent phosphorylation. After activation, these Smad proteins form a heteromeric complex with Smad 4 and that complex is translocated to the nucleus where it regulates transcription of specific genes. The third group consists of Smad 6 and Smad 7, which, despite structural homology, are negative regulators for the TGF β1 signalling (Heldin *et al.*, 1997; Massague, 1998). Lack of Smad 4 expression or function has been frequently observed in the pancreatic (Hahn *et al.*, 1996) and colorectal (Thiagalingam *et al.*, 1996) cancers and to a lower extent in seminoma, juvenile polyposis, esophageal, head and neck, ovarian, lung, breast and biliary tract cancers (for review see Souchelnytskyi, 2002). The low incidence of cancer-related mutations of Smad 2 and Smad 3 genes is in contrast with the high frequency of Smad 4 gene mutations (for a review see Souchelnytskyi, 2002).

Clinically, TGF β1 is often elevated in the plasma of breast, lung, and prostate cancer patients and in hepatocellular carcinoma patients. Preclinically, several breast cancer and prostate cancer models *in vivo* have demonstrated a connection between TGF β1 expression and increased tumorigenicity, increased invasion and drug resistance (Teicher, 2001). In other oncological diseases such as colon, gastric, endometrial, ovarian, and cervical cancers, and gliomas and melanoma, loss of response to TGF β1 as a growth inhibitor, and increased expression of TGF β1 have been associated with malignant conversion and progression (Teicher, 2001). Elevated levels of

TGF β1 are measurable in nude mice bearing a wide variety of human tumor xenografts (Teicher, 2001). Overexpression of TGF β1 has been reported in human breast carcinoma resistant to the anti-estrogen tamoxifen, and TGF β1-overexpressing tumors in nude mice treated with tamoxifen plus TGF β1 antibodies failed to grow, whereas tumors treated with tamoxifen plus a control antibody continued to proliferate (Arteaga *et al.*, 1999). However, data also exist about a positive correlation between a decline in plasma TGF β1 levels and advancing chronic lymphocytic leukemia stage (Fridenberg *et al.*, 1999). Thus, potential diagnostic and/or prognostic roles of TGF β1 level in cancer patients are still to be cleared up.

It is known that long term chemotherapy induces drug resistance in many cancer patients (Young, 1989). However, up to now, no distinct mechanism(s) have been proposed to explain the interrelations between the appearance of refractoriness to an anti-cancer drug and to TGF β1 in tumor cells. Here we propose a novel mechanism for drug resistance based on the involvement of TGF β1 – an ubiquitous cytokine which can inhibit proliferation of many target cells and cause their apoptosis (Heldin *et al.*, 1997; Massague, 1998). This mechanism is based on the co-expression of tumor cell refractoriness to both an anti-cancer drug and TGF β1. We found that cisplatin-resistant derivatives of murine L1210 leukemia cells are not susceptible to the negative actions of TGF β1. While the cisplatin-sensitive cells possessed intact TGF β1 signal transduction pathway, their cisplatin-resistant derivatives were characterised by a decreased expression of type I TGF β1 receptors and increased expression of the post-receptor Smad 6 protein inhibiting TGF β1 signal transduction. Taking into account that several anti-cancer drugs cause an increase in TGF β1 expression by L1210 leukemia cells, we suggest that the impairment in TGF β1 signalling in these cells could be at least partly responsible for their cisplatin resistance. The potential molecular mechanisms for TGF β1-dependent development of drug resistance in tumor cells are discussed.

MATERIALS AND METHODS

Cell line and culture conditions. Murine leukemic cells L1210 (parental cisplatin-sensitive and derivative cisplatin-resistant lines) used in this study were obtained from the Cell Collection at the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, U.S.A.), supplemented with 10% fetal calf serum (FCS; Sangva, Lviv, Ukraine).

Drugs, reagents and kits. The anti-cancer drugs used in this study were bought in local pharmacies and were produced by Bristol, Germany (cisplatin), Ebewe, Austria (methotrexate), and Faulding Pharmaceuticals, U.S.A. (vincristine sulfate). They were dissolved in accordance with the drug description and then added to the culture medium as indicated in the experiment protocols. TGF β1 was purchased from R & D Systems (Indianapolis, IN, U.S.A.). The kit DB100 for TGF β1 ELISA detection was from R & D Systems (Minneapolis, MN, U.S.A.). Mistletoe (*Viscum album*) agglutinin was isolated and purified to electrophoretic homogeneity in our laboratory by Dr. M. Lutsik as described (Khomutovsky *et al.*, 1986). Reagents used for Western blot analysis and for DNA fragmentation study were from Sigma.

Antibodies. Antisera raised against peptides derived from the linker regions of Smad 2, Smad 3, and Smad 4 were described by (Nakao *et al.*, 1997). Antisera against Smad 6 and Smad 7 were raised against the specific synthetic peptides at the Ludwig Institute for Cancer Research (Uppsala, Sweden). Antiserum against the phosphorylated C-terminus of Smad 2 has beed described by (Persson *et al.*, 1998). DRL antiserum recognizes differ-

ent isoforms of type II receptor for TGF β1 and VPN antiserum was prepared against the juxta-membrane region of type I receptor for TGF β1 using a specific peptide. Each antiserum was found to be specific for respective Smad proteins, and not to cross-react with other Smads (Korchynskyi *et al.*, 1999).

Determination of cell number and Trypan blue staining for cytotoxicity assay. The cell number and the proportion of dead cells was determined in the presence of 0.01% (w/v) Trypan blue solution by counting stained (dead) and unstained (alive) cells in a hemocytometer camera under a light microscope.

DNA preparation and electrophoresis. An earlier described method was used (Herrmann *et al.*, 1994). Briefly, 5×10^6 cells were pelleted and resuspended in 50 μl of 20 mM EDTA/50 mM Tris/HCl, pH 7.5, centrifuged for 5 min at 1600 × ***g*** and pellets were resuspended in lysis buffer. SDS (final concentration 1%) and RNase A (final concentration 1 mg/ml) were added to each sample which were then incubated for 1 h at 37°C. After that, proteinase K (final concentration 1 mg/ml) was added to each sample which was then incubated for 1 h at 37°C. Then 10 M ammonia acetate (50% of sample volume) was added to each sample and DNA was precipitated with 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 dry wells of 1% (w/v) agarose gel. Electrophoresis was carried out in 1 mM EDTA/40 mM Tris/acetate buffer, pH 8.0, until the marker dye migrated 6–7 cm. Electrophoregrams were stained with ethidium bromide, screened on a transilluminator under UV light and photographed.

Western blot analysis. L1210 cells were grown and treated with TGF β1 (5.0 ng/ml), cisplatin (0.1 μg/ml for L1210/S parental cells and 1.0 μg/ml L1210/R cisplatin-resistant derivatives, 24 h) or VAA-1 (5 ng/ml, 24 h). The cells were washed with cooled phosphate-buffered saline and then collected and solubilized in a buffer containing 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1% Trasylol, 1 mM PMSF. After 20 min incubation on wet ice, the cell lysates were cleared up by centrifugation, boiled for 5 min with SDS sample buffer (100 mM Tris/HCl, pH 8.8, 0.01% bromphenol blue, 36% glycerol, 4% SDS, 10 mM DTT) and subjected to SDS/PAGE electrophoresis. Protein fractions were then electrotransfered to nitrocellulose membrane, immunoblotted with anti-Smad antibody and developed using the enhanced chemiluminescence detection reagents. For detection of expression of TGF β1 receptors membrane fraction of L1210 cells was isolated. Briefly, washed cells were suspended in a hypotonic buffer (10 mM Tris/HCl, pH 7.5, 1.5 mM $MgCl_2$, 1 mM PMSF, 1 mM benzamidine), left for 10 min on wet ice, and then homogenized using a Potter type homogenizer. An appropriate volume of 2.0 M sucrose was added immediately to the homogenate to a final concentration of 0.25 M and centrifugation was carried out for 15 min at 2000 × ***g*** to pellet the nuclei and intact cells. The pellets were homogenized once more in the above hypotonic buffer. The supernatants originating from three homogenization procedures were combined and centrifuged for 60 min at 30 000 × ***g***. All operations were performed at 4°C. The resulting membrane proteins were solubilized in SDS sample buffer, boiled for 5 min, subjected to SDS/PAGE, and electrotransfered to nitrocellulose membrane. TGF β1 receptors were detected on the membrane with specific polyclonal anti-TβRI (VPN) or anti-TβRII (DRL) antibodies and with the enhanced chemiluminescence detection reagents.

ELISA detection of TGF β1. L1210 cells were grown to the sub-confluent state, and the culture medium was changed for serum free one for 24 h. Then fresh serum-free medium was added together with TGF β1 or other studied agents for another 24 h. The collected conditioned media were studied using ELISA

TGF β1 detection kit. Before use the conditioned media were acidified with HCl to pH 2.0 for 1 h at room temperature and then the pH was brought to neutral with NaOH. All manipulations were then carried out in accordance with the ELISA kit protocol.

Statistical analysis. Experiments were performed in triplicate and repeated 3 times. The significance of the difference in a typical experiment was assessed by Student's *t*-test. The level of significance was set at 0.05.

RESULTS

Proliferation of cisplatin-sensitive and cisplatin-resistant L1210 cells under TGF β1 effect

We found a distinct difference between the TGF β1 effects on the proliferation of two variants of murine L1210 leukemia cells – sensitive (L1210/S) and resistant (L1210/R) to cisplatin. TGF β1 was an effective growth inhibitor for L1210/S cells and did not affect the growth of L1210/R cells (Fig. 1). TGF β1 concentration of 5 ng/ml was used in this experiment as it was ealier found (Stoika *et al.*, 1999) that L1210/S cells were strongly inhibited while L1210/R cells were only slightly affected in the presence of such TGF β1 concentration. Thus, a cross-resistance to cisplatin and the inhibiting cytokine TGF β1 exists in these leukemia cells. It was also detected that the L1210/R cells grew slower in the experimental conditions used (Fig. 1), although it is known that drug-resistant cell phenotype is usually associated with higher cell malignancy (Young, 1989; Shishova & Chekhun, 1998; Waxman, 1990; Krishan *et al.*, 1997; Brown & Woulters, 1999).

Apoptosis of cisplatin-sensitive and cisplatin-resistant L1210 cells under TGF β1 effect

Data exist that both cisplatin (Chu, 1994) and TGF β1 (Heldin *et al.*, 1997; Massague, 1998) can induce apoptosis in tumor cells, including cisplatin-sensitive L1210 cells (Motyl *et al.*, 1996). Here we show that TGF β1 in concentration of 5 ng/ml is ineffective in the induction of apoptosis in cisplatin-resistant L1210 cells (Fig. 2). It should be noted that DNA laddering in L1210 cells is always accompanied by the expression of other apoptotic markers (Segal-Bendirdjian *et al.*, 1998). Such an effect of TGF β1 on apoptosis did not depend on FCS concentration, as apoptosis in L1210/S cells was observed in both presence and absence of FCS in culture medium (not shown).

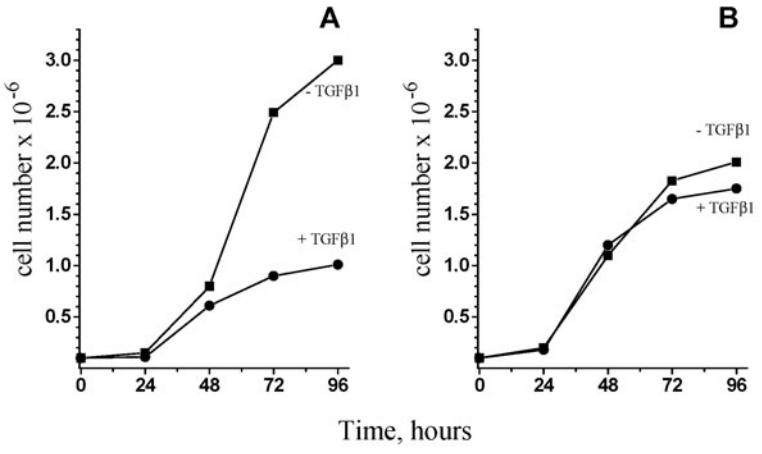

Figure 1. Effect of TGF β1 on the proliferation of L1210/S and L1210/R cells.

L1210/S (**A**) or L1210/R (**B**) 10^5 cells were cultured in 5% FCS-supplemented DMEM for different times in the absence or presence of TGF β1 (5 ng/ml).

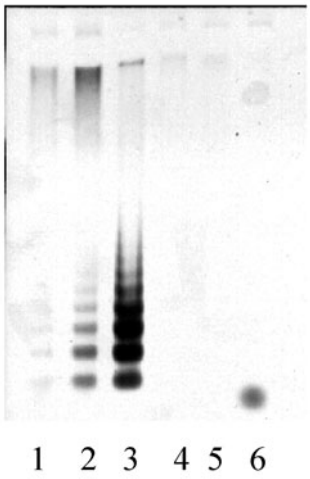

Figure 2. Effect of cisplatin and TGF β1 on DNA fragmentation (apoptosis) in L1210/S and L1210/R cells.

DNA was prepared from cells cultured for 24 h in FCS-free DMEM before cisplatin or TGF β1 addition for 48 h in the presence of 2% FCS-supplemented DMEM. 1 and 4, untreated L1210/S and L1210/R cells, respectively; 2 and 5, TGF β1 (5 ng/ml)-treated L1210/S and L1210/R cells, respectively; 3 and 6, cisplatin (1 μg/ml)-treated L1210/S and L1210/R cells, respectively.

TGF β1 production by cisplatin-sensitive and cisplatin-resistant L1210 cells under the effect of antitumor drugs and cytotoxic lectins

To address the question about the mechanisms responsible for the development of refractoriness of L1210/R murine leukemia cells to TGF β1-dependent inhibition of proliferation and induction of apoptosis we studied: 1) TGF β1 production by L1210/S (TGF β1-sensitive) and L1210/R (TGF β1-resistant) cells; 2) expression of two types of TGF β receptors – TβRI and TβRII; 3) expression of Smad 2, 3, 4, 6, and 7 proteins involved in post-receptor TGF β1 signalling; 4) expression of the anti-mitogenic and the pro-apoptotic p53 and the pro-mitogenic and anti-apoptotic Bcl-2 proteins. In all the above mentioned experiments the L1210/S and L1210/R cells were studied in parallel. Besides, the effect of different apoptosis-inducing agents (TGF β1, anti-cancer drugs and cytotoxic plant lectins) was studied in both variants of L1210 cells.

We show that L1210/R cells produce higher amount of TGF β1 than L1210/S cells (Fig. 3). A similar difference between cisplatin-sensitive and cisplatin-resistant tumor cells was observed by other investigators (Teicher *et al.*, 1997). It should be noted that a detectable TGF β1 amount was revealed only after temporal acidification of the conditioned media after L1210 cells. It is known that such procedure activates TGF β1 *via* releasing the masking protein(s) from latent complex(es) (Heldin *et al.*, 1997; Massague, 1998). Both L1210/S and L1210/R cells were induced by the apoptotic agents to TGF β1 production, however, the character of the action of different agents was specific for the target cells. When L1210/S cells were tested, cisplatin and the

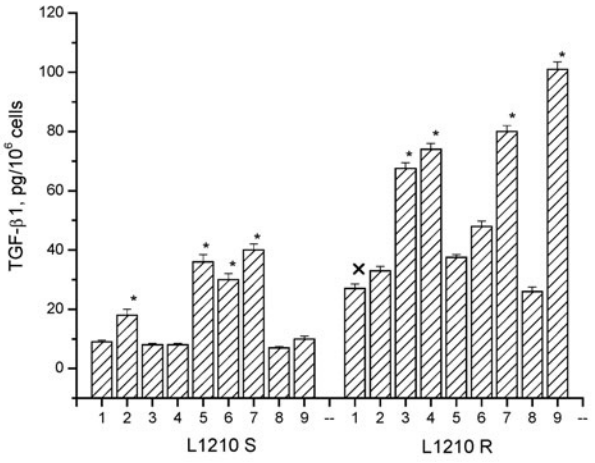

Figure 3. Effect of antitumor drugs and cytotoxic lectins on TGF β1 production by L1210/S and L1210/R cells.

L1210/S or L1210/R cells were cultured for 24 h in FCS-free DMEM before anti-cancer drug or cytotoxic lectin addition for 48 h in the presence of fresh FCS-free DMEM. ELISA detection of TGF β1 was performed in the collected conditioned media. **1**, no treatment; **2**, cisplatin (1 μg/ml); **3**, methotrexate (10 μg/ml); **4**, vincristine (0.1 μg/ml); **5**, VAA-1 (5 ng/ml); **6**, VAA-2 (5 ng/ml); **7**, RCA-120 (5 ng/ml); **8**, Con A (10 μg/ml); **9**, WGA (2 μg/ml). $\times$, $P < 0.05$ (L1210/R *versus* L1210/S cells) $\ast$, $P < 0.05$ (cells treated by different agents *versus* untreated cells).

cytotoxic lectins VAA-1, VAA-2 and RCA-120 induced TGF β1 production, while when L1210/R cells were tested, methotrexate, vincristin, and the cytotoxic lectins RCA-120 and WGA induced TGF β1 production (Fig. 3). Only statistically meaningful differences were mentioned above. Con A, which is known to be relatively non-toxic, did not affect TGF β1 production by the studied cell lines. Thus, TGF β1-resistant L1210 cells possess a capability of increased production of TGF β1. Besides, different cytotoxic agents whose character depended on TGF β1 resistance of the target cells induced these leukemic cells to TGF β1 production.

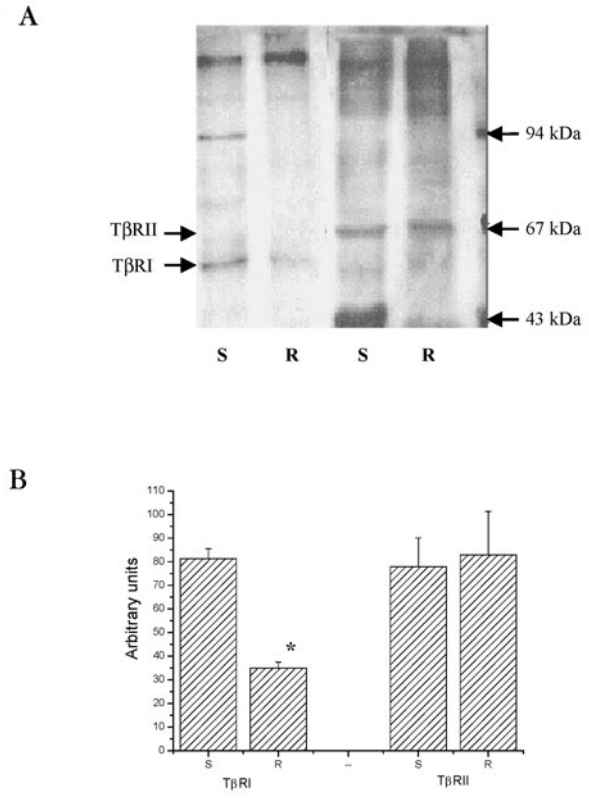

Figure 4. Expression of TβRI and TβRII (type I and type II TGF β1 receptors, respectively) in L1210/S and L1210/R cells.

A, L1210/S (S) and L1210/R (R) cells were grown in 10% FCS-supplemented DMEM and cell membranes were prepared as described in (Khomutovsky *et al.*, 1986). Cell membrane lysates were prepared for Western blot analysis as described in Materials and Methods. **B**, Densitometric measurement of TβRI and TβRII proteins in L1210/S (S) and L1210/R (R) cells. ⁎, $P < 0.05$ (L1210/S *versus* L1210/R cells).

Expression of TGF β1 receptors in cisplatin-sensitive and cisplatin-resistant L1210 cells

In the next two experiments the expression of specific components of the TGF β1 signalling pathway (TGF β1 receptors type I and II and different post-receptor signalling Smad proteins) was compared in L1210/S and L1210/R cells. Using VPN antibodies which specifically recognize the TβRI protein we revealed a significant decrease in its expression in L1210/R cells comparing with L1210/S cells (Fig. 4). At the same time, the TβRII protein was well expressed in both L1210/S and L1210/R cells. The specificity of both these antibodies was proven in previous studies, with controls of over-expressed exogenous receptors. These antibodies showed major immunoreactivity at the expected migration positons. Thus, the increased resistance of L1210/R cells to the growth-inhibiting and apoptosis-inducing effects of TGF β1 could be caused by a decreased expression of type I TGF β1 receptors.

Expression of different Smad proteins in cisplatin-sensitive and cisplatin-resistant L1210 cells

It is known that activated TGF β1 receptors transduce their regulatory signal(s) *via* specific Smad 2, 3, and 4 proteins, while Smad 6 and 7 proteins can block TGF β1 intracellular signalling (Heldin *et al.*, 1997; Massague, 1998; Souchelnytskyi, 2002). One can see in Fig. 5A that there is no marked difference in the expression of Smad 2, 3, and 4 between L1210/S and L1210/R cells. The cytokine TGF β1, the anti-cancer drug cisplatin, or the cytotoxic plant lectin VAA-1, did not affect the expression of those Smads (Fig. 5A) in the studied cell lines. It has been shown that the above mentioned agents induce apoptosis in

L1210/S cells (Motyl *et al.*, 1996; Stoika *et al.*, 1999; Yakymovych *et al.*, 1999).

We also studied the expression of phosphorylated Smad 2 because it is known that such modification is necessary for realization of its signal transduction function (Heldin *et al.*, 1997; Massague, 1998; Souchelnytskyi, 2002). We found distinct induction of the expression of such form of the Smad 2 protein under TGF β1 action in both L1210/S and L1210/R cells (Fig. 5A). However, no phosphorylated Smad 2 was detected under the action of cisplatin or the cytotoxic lectin VAA-1 on L1210/S or L1210/R cells. That proves the absence of the active form of TGF β1 in the conditioned media collected after L1210 cells treated with various proapoptotic agents. The antibodies which we used for the detection of phosphorylated Smad 2 did not reveal phosphorylated Smad 3, which is known to be phosphorylated together with Smad 2 (Heldin *et al.*, 1997; Massague, 1998).

The level of expression of the inhibitory Smad 6 was found to be elevated in L1210/R cells comparing with L1210/S cells (Fig. 5B). Another inhibitory protein, Smad 7, was expressed at a similar level in both studied cell lines (Fig. 5A). It is noteworthy that TGF β1 and VAA-1 induced the expression of Smad 6 in L1210/S cells to the levels comparable to those in L1210/R cells (Fig. 5B). The level of expression of the Smad 6 protein was not changed under the action of TGF β1 or VAA-1 in L1210/R cells (Fig. 5B).

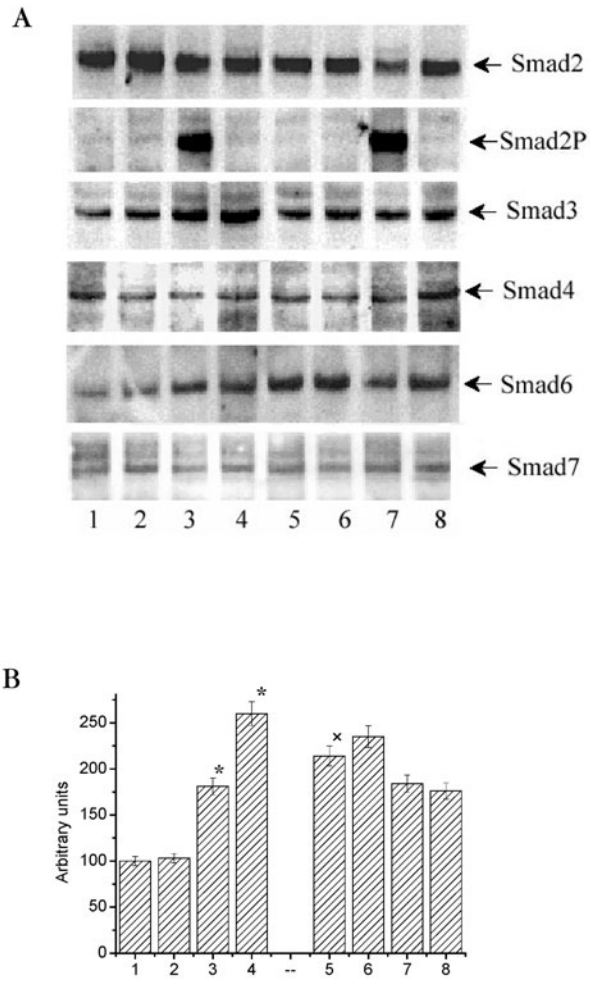

Figure 5. Expression of Smad proteins in L1210/S and L1210/R cells.

A, L1210/S (1–4) and L1210/R (5–8) cells were grown in 10% FCS-supplemented DMEM and cell lysates were prepared for Western blot analysis as described in Materials and Methods. **B**, Densitometric measurement of Smad 6 protein in L1210/S (1–4) and L1210/R (5–8) cells. 1 and 5, untreated cells; 2 and 6, cisplatin (1 μg/ml)-treated cells; 3 and 7, TGF β1 (5 ng/ml)-treated cells; 4 and 8, VAA-1 (5 ng/ml)-treated cells. ×, $P < 0.05$ (L1210/R *versus* L1210/S cells); ⁕, $P < 0.05$ (treated L1210/S cells *versus* untreated L1210/S cells).

Expression of p53 and Bcl-2 proteins in cisplatin-sensitive and cisplatin-resistant L1210 cells

As mentioned above, there is a big difference between L1210/S and L1210/R cells in their susceptibility to cisplatin- and TGF β1-induced apoptosis. Thus, it was reasonable to compare the expression of the pro-apoptotic p53 and anti-apoptotic Bcl-2 proteins in those cell lines. We found a loss of p53 expression and elevated Bcl-2 expression in the L1210/R comparing with L1210/S cells (Fig. 6). Thus, multiple disturbances in regulatory systems appear in cisplatin- and TGF β1-resistant murine leukemia L1210 cells.

DISCUSSION

Here we describe a cross-resistance to cisplatin and to TGF β1 in murine leukemia L1210 cells, which are a convenient experimental model for studying the mechanisms of tumor cell drug resistance (Segal-Bendirdjian *et al.*, 1998). We showed that both proliferation and apoptosis in L1210/R cells were relatively insensitive to the TGF β1 effect.

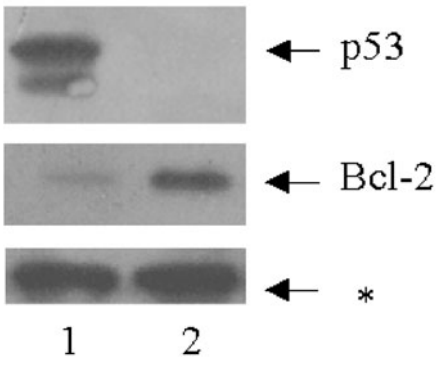

Figure 6. Expression of p53 and Bcl-2 proteins in L1210/S and in L1210/R cells.

L1210/S (**1**) and L1210/R (**2**) cells were grown in 10% FCS-supplemented DMEM and cell lysates were prepared for Western blot analysis as described in Materials and Methods; *, non-specific protein detected with polyclonal antiserum.

Defects at the TGF β1 receptor and post-receptor levels may be responsible for cell refractoriness to TGF β1 action. Thus, it could be predicted that at least one of these levels is changed in L1210/R cells. We revealed a significant decrease in the expression of type I TGF β1 receptor, which is probably enough to stop TGF β1 signalling in L1210/R cells. Both TβRI and TβRII deteriorations are characteristic in many specific cancers (for a review see Souchelnytskyi, 2002). However, ovarian carcinoma cell cultures were shown to be resistant to TGF β1-mediated growth inhibition despite expression of functional receptors for this cytokine (Yamada *et al.*, 1999). That means that other mechanisms of growth resistance down-stream of TβRI phosphorylation may be important for the development of TGF β1 resistance.

We found that L1210/R cells have elevated expression of the inhibitory Smad 6 which is known to block TGF β signalling (Heldin *et al.*, 1997; Massague, 1998). Implication of the inhibitory Smad 6 and Smad 7 production in tumorigenesis has been less explored than that of Smads 2, 3 and 4. An increased expression of Smads 6 and 7 was shown in pancreatic cancers, although no cancer-related mutations in their genes were reported (Kleeff *et al.*, 1999a; 1999b). The results of our analysis do not allow us to identify the mechanism(s) responsible for the elevated Smad 6 expression in cisplatin- and TGF β1-resistant murine leukemia L1210 cells.

Taking into account the data about the resistance of L1210/R cells to the apoptosis-inducing effects of cisplatin or TGF β1, it was reasonable to compare the expression of the pro-apoptotic p53 and anti-apoptotic Bcl-2 proteins in L1210/S and L1210/R cells. As predicted, L1210/R cells were characterized by a lack of expression of p53 and an elevated expression of Bcl-2. However, data exist showing that a default in apoptosis in L1210 cisplatin-resistant cells did not result from differential expression of the anti-apoptotic protein Bcl-2 or from altered expression of p53 (Canitrot *et al.*, 1997). Evidence also has been presented that deficiency in the p53 pathway and resistance to DNA-damaging agents due to a defect in apoptosis are independent events (Segal-Bendirdjian *et al.*, 1998).

Thus, the lack of response to TGF β1 action in L1210/R cells could be explained by multiple changes which accompany the development of cisplatin resistance in these tumor cells. These changes involve: 1) loss of TGF β1 receptor; 2) elevated expression of Smad 6 protein blocking TGF β signalling; 3) loss of the pro-apoptotic p53 protein and elevated expression of the anti-apoptotic Bcl-2 protein. The presented data prove that multiple changes in cell regulatory systems should take place during malignant transformation and they lead to a loss of normal cell response to the action of growth, differentiation and

apoptosis regulators. It is evident that changes in the functioning of the regulatory system of TGF β which often acts as a growth inhibitor and apoptosis inducer can play an important role in oncogenesis.

A comparative study of TGF β1 expression in L1210/S and L1210/R cells was carried out. It is known that in most cases TGF β1 produced stays in a latent form in which it is complexed with specific "masking" protein(s) (Heldin *et al.*, 1997; Massague, 1998). *In vitro*, the release of biologically active TGF β1 is most easily performed in acidified medium. Using ELISA we did not find a significant production of TGF β1 by either L1210/S or L1210/R cells (not shown). However, temporary acidification of conditioned media according to the manufacturer's protocol for the TGF β1 detection kit revealed higher expression of TGF β1 by L1210/R cells comparing with L1210/S cells.

Our data are in agreement with the results of an *in vivo* study showing that mice bearing cisplatin-resistant mammary tumors have higher plasma levels of TGF β1 than animals bearing the parent ones (Teicher *et al.*, 1997). Besides, it was found that upon treatment with cytotoxic therapies there is a greater rise in plasma TGF β1 levels in animals bearing the parent tumor than in animals bearing the cisplatin-resistant ones (Teicher *et al.*, 1997). Our *in vitro* studies on the effect of anti-cancer drugs and cytotoxic lectins on TGF β1 expression by L1210/S and L1210/R cells revealed that both variants of L1210 cells were responding to the action of various stressing agents by an increase in TGF β1 expression. However, such response by L1210/S cells differed from the response of L1210/R cells to a specific drug or lectin. It looks that only negatively acting agents possess an ability to induce TGF β1 expression in the target cells, because the relatively non-toxic lectin Con A did not affect L1210/S or L1210/R cells.

Thus, although TGF β1 is produced by both L1210/S and L1210/R cells mostly in its latent form, the refractoriness to this inhibiting cytokine in L1210/R cells could be caused by the elevated expression of the Smad 6 protein blocking TGF β signalling. It is noteworthy that both TGF β1 and the cytotoxic mistletoe agglutinin cause an increase in Smad 6 expression. While in the case of TGF β1 action that effect could be explained by a negative regulation of an excess cytokine activity, the increased Smad 6 expression under cytotoxic agglutinin action on L1210/S, but not on L1210/R cells, suggests a potential mechanism how the stressing drugs could cause TGF β1 resistance. This hypothesis is presently under investigation.

The hypothetical sheme presented in Fig. 7 explains how anti-cancer drugs may mediate their action through the TGF β1-dependent pathway, and how the appearance of TGF β1 resistance may partially block the effect(s) of specific anti-cancer drugs. Thus, it is suggested that in TGF β1-sensitive cancer cells specific antitumor drugs induce TGF β1 expression either at the level of transcription of its gene, translation of its mRNA, or secretion of the protein. After activation of its latent form TGF β1 acts through specific cell receptors in an autocrine or paracrine manner to inhibit cell growth or induce apoptosis. This mechanism increases the negative effect of the antitumor drug on target cells. Parallely, TGF β1 acts as an immunosuppressing cytokine (for a review see Heldin *et al.*, 1997; Massague, 1998) and it is known that many antitumor drugs possess an immunosuppressing activity.

In the case when TGF β1 resistance develops in cancer cells, the TGF β1-dependent growth inhibition or apoptosis induction are blocked, while the immunosuppressive action of TGF β1 is preserved. The aggressivenes of TGF β1-resistant tumors is known to be increased (for review see Souchelnytskyi, 2002). An experimental proof of the above described mechanism could serve for grounding a novel mechanism for tumor cell drug resistance involving a potential role of TGF β1 in that mechanism.

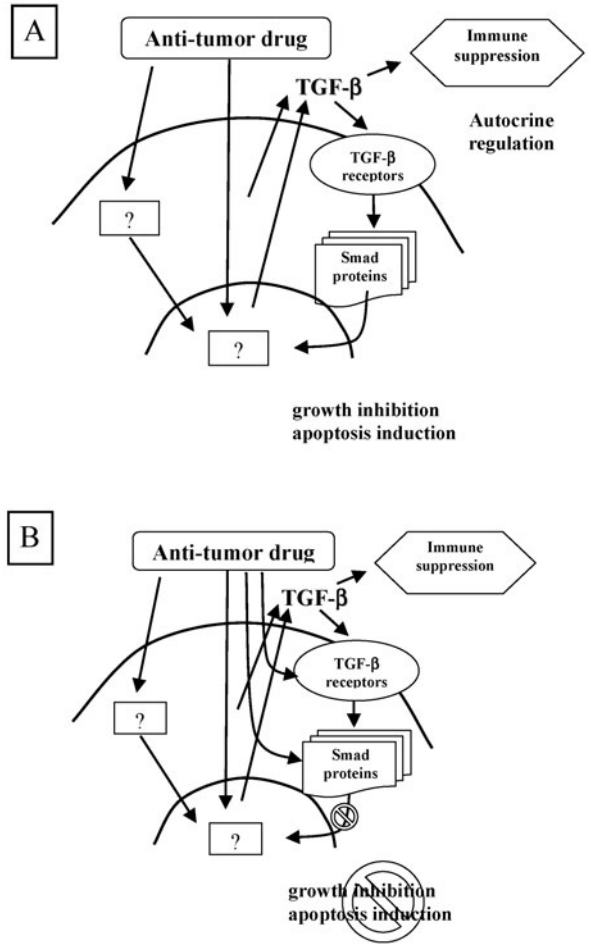

Figure 7. Hypothetical scheme for TGF β1 involvement in anti-cancer drug action (A) and in drug resistance, dependent on the impairment of TGF β1 signal transduction pathway (B).

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