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Report

Cisplatin-resistant derivatives of murine L1210 leukemia cells are not susceptible to growth-inhibiting and apoptosis-inducing actions of transforming growth factor-β1

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Murine L1210 leukemia cells possessing an increased resistance to cisplatin were found to be refractory to transforming growth factor (TGF)-β1-induced growth inhibition, while the parental L1210 cells were strongly inhibited by this cytokine. Growth inhibition was estimated on the basis of [^{3}H]thymidine incorporation, cell counting and colony-forming assay. Cisplatin-resistant L1210 cells were also shown to be much more resistant than the parental cells to both cisplatin- and TGF-β1-induced apoptosis. These results suggest the existence of cross-resistance to cisplatin and TGF-β1 in the studied leukemia cells. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cisplatin, growth inhibition, L1210 leukemia cells, resistance, transforming growth factor-β1.

Introduction

Acquired resistance to cisplatin is a major clinical problem in the treatment of cancer, particularly ovarian, testicular, and head and neck carcinomas.[1] Different biochemical mechanisms have been proposed to produce cisplatin resistance: (i) decreased cellular accumulation of cisplatin, (ii) increased levels of glutathion or of glutathion-S-tranferase activity, (iii) increased levels of intracellular metallothioneins and (iv) enhanced DNA repair.[1,2] However, up to now, none of these mechanisms have been accepted as universal or dominating.

Transforming growth factor (TGF)-β1 belongs to a large superfamily of cytokines that includes more than 40 members, and that regulate cell growth, differentiation, migration, matrix production and apoptosis.[3,4] A positive correlation was found between the malignant potential of the tumor cells and their resistance to TGF-β1-induced growth inhibition.[5] TGF-β1 was shown to inhibit the proliferation of irradiated murine 10T1/2 cells which developed into benign tumors in mice and gave no lung metastases. At the same time, the proliferation of T24-Ha-*ras*-transfected cells developing into malignant tumors with numerous lung metastases was stimulated by TGF-β1.[5] Of the 13 studied patients with chronic lymphocytic leukemia, cells isolated from peripheral blood of eight patients were shown to be sensitive to growth inhibition by TGF-β1, whereas those from five patients were completely resistant to TGF-β1 action.[6]

At present there is no uniformity in the results of the study of the inter-relation between the action of TGF-β1 and cisplatin on malignant cells. There is evidence that TGF-β1 enhances the lethal effects of cisplatin and of other DNA-damaging agents (UV and γ rays, methotrexate and 5-fluorouracil) toward human lung adenocarcinoma cells of the A-549 line, as detected by the loss of their capacity to give rise to colonies and develop apoptosis.[7] TGF-β1 and cisplatin induced apoptosis in human erythroleukemia K562 cells in an additive manner.[8] Other authors,[9] using rat embryo fibroblast lines expressing different p53 mutations, concluded that reversible inhibitors of the cell cycle

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such as TGF-β protect dividing cells from exposure to anticancer drugs and radiation as shown by colony-forming assays. Treatment of tumor-bearing animals with anti-TGF-β1 antibodies increased the sensitivity of tumor to cyclophosphamide and cisplatin action.

Murine leukemia cells of the L1210 line were used in different laboratories[1,2,10,11] for the study of development of tumor cell resistance to the action of cisplatin. Our study has been performed in order to investigate whether the development of resistance to cisplatin in L1210 cells may interfere with their sensitivity to TGF-β1. The results obtained suggest that cisplatin-resistant derivatives of L1210 cells are not susceptible to the growth-inhibiting and apoptotic actions of TGF-β1 in contrast to cisplatin-sensitive parental cell line.

Materials and methods

Cell line and culture conditions

Murine leukemic cells of L1210 (parental and cisplatin-resistant lines) used in this study were obtained from Cell Collection at the 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), supplemented with 10% fetal calf serum (FCS; Sangva, Lviv, Ukraine).

Drugs

Cisplatin [*cis*-diamminedichloroplatinum(II)] was purchased from Bristol (Munchen, Germany). It was dissolved in 0.15 M NaCl and then added to the culture medium as indicated in the experiment protocols. TGF-β1 was purchased from R & D Systems (Indianapolis, IN).

Trypan blue staining and determination of cell number

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 and unstained cells under the light microscope.

[^{3}H]thymidine incorporation

Aliquots of 20 μl cisplatin or TGF-β1 were placed in 24-well plates containing 10^4 L1210/S (parental cisplatin-sensitive cells) or L1210/R (cisplatin-resistant) cells per well in 200 μl of assay buffer (DMEM containing 5% FCS in the case of TGF-β1 assay and DMEM containing 10% FCS in the case of cisplatin assay). After 20 h at 37°C, 2 μCi of [^{3}H]thymidine (50 Ci/mmol) in 20 μl of the assay buffer was added to each well and plates were incubated for an additional 4 h. The cells were harvested, transfered to Eppendorf tubes and washed 2 times with ice-cold PBS. The amount of radioactivity was quantified in TCA-insoluble materials with a β-scintillation counter (Pharmacia LKB, Uppsala, Sweden).

Colony-forming assay

The colony-forming assay was performed essentially as described.[12] Briefly, 10^4 cells were seeded in 1 ml of the culture medium with 0.3% agar (Difco, Detroit, MI) per 40 mm plastic dish, precovered with 1 ml of the culture medium containing 0.5% agar. In parallel experiments, TGF-β1 (5 ng/ml) or cisplatin were also added to the culture medium (see legends of figures). The cells were incubated for 2 weeks in a CO_2 incubator and cell colonies more than 50 μm in diameter were counted.

DNA preparation and electrophoresis

These were performed as described.[13] Aliquots of 5×10^6 cells were pelleted and resuspended in 50 μl of 20 mM EDTA/50 mM Tris-HCl (pH 7.5) containing 1% NP-40 (lysis buffer). Samples were centrifuged for 5 min at 1600 *g* and pellets were resuspended in lysis buffer. SDS (Serva, Heidelberg, Germany; final concentration 1%) and RNase A (Sigma St Louis, MO; final concentration 1 mg/ml) were added to each sample which was then incubated for 1 h at 37°C. After that, proteinase K (Sigma; 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 (Sigma; 50% of the 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*, and pellets were dried, dissolved in TE buffer (10 μl/10^6 cells) and loaded into the dry wells of 1% (w/v) agarose gel. Electrophoresis was carried out in 0.001 M EDTA (Serva)/0.04 M Tris-acetate buffer (pH 8.0) until marker dye migrated 6–7 cm. Electrophoregrams were examined in a transilluminator (Hoefer Pharmacia Biotech, San Francisco, CA) under UV light and photographed.

Statistical analysis

Each experiment was performed in triplicate and repeated 3 times. 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

Three different tests were used for the study of effects of cisplatin and TGF-β1 on the growth of murine L1210 leukemia cells: (i) [^{3}H]thymidine incorporation (Figures 1 and 2), (ii) the change in cell number (Figures 3 and 4) and (iii) the colony formation by the cells grown in 0.3% agar-supplemented culture medium (Figure 5). Two variants of cells were used in each of these tests: (i) parental L1210 cells, which are sensitive to the inhibitory action of cisplatin (L1210/S), and (ii) selected L1210 cells possessing an increased resistance to such inhibition (L1210/R).

As can be seen from Figure 1, a clear difference exists between two variants of cells in their sensitivity to cisplatin effect. Dose-dependent inhibition of [^{3}H]thymidine incorporation under cisplatin action could be seen in L1210/S cells, while the L1210/R cells were lacking this effect. The same conclusion could be made on the basis of the analysis of TGF-β1 effect (Figure 2).

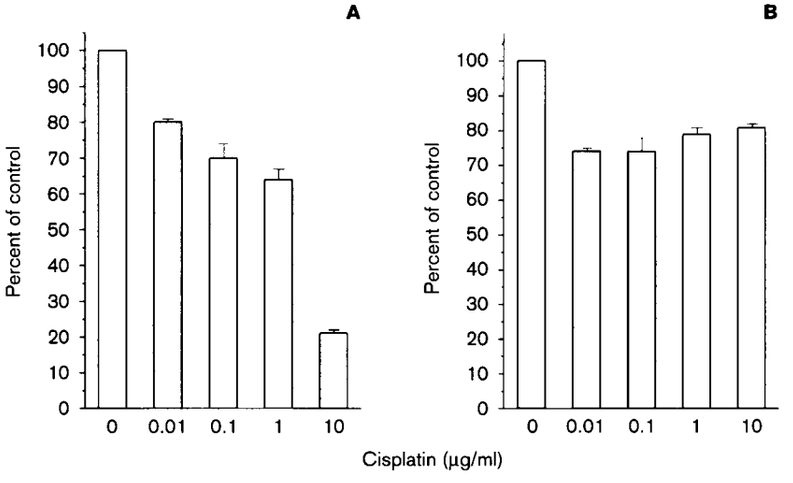

Figure 1. Effect of cisplatin on [^{3}H]thymidine incorporation into DNA of L1210/S (A) and L1210/R (B) cells. Ordinate: 100% for A was 105 000 c.p.m., for B was 86 000 c.p.m.

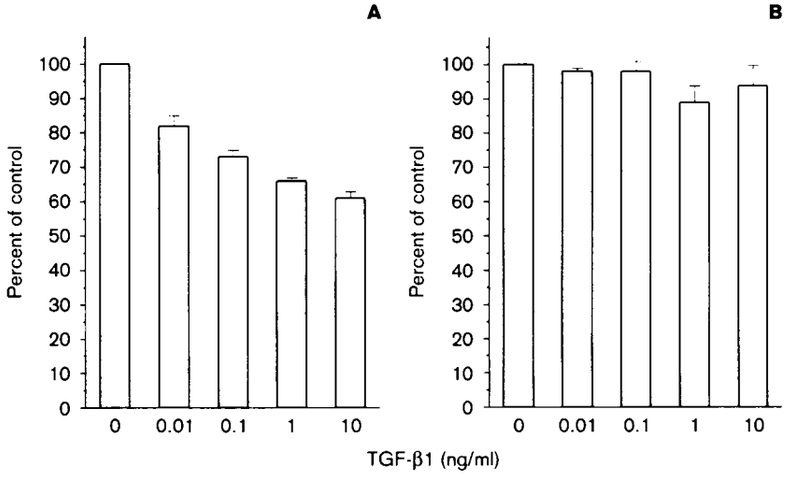

Figure 2. Effect of TGF-β1 (5 ng/ml) on [^{3}H]thymidine incorporation into DNA of L1210/S (A) and L1210/R (B) cells. Ordinate: 100% for A was 103 000 c.p.m., for B was 67 000 c.p.m.

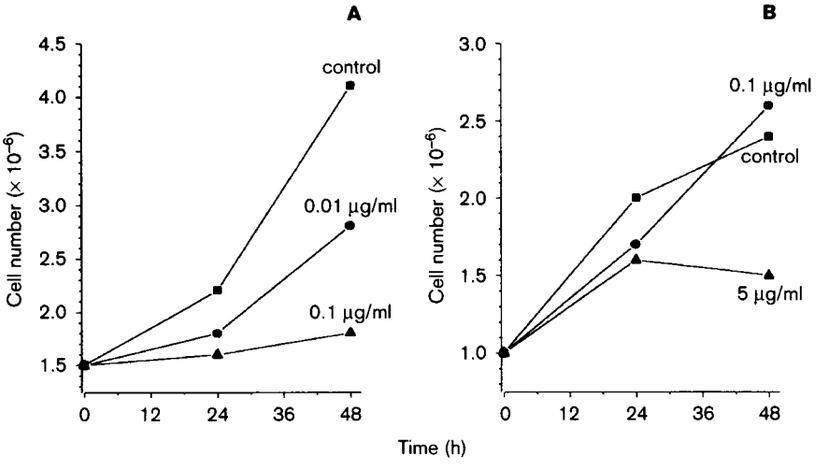

Figure 3. Effect of cisplatin on the growth of L1210/S (A) and L1210/R (B) cells (final concentration of added cisplatin is indicated).

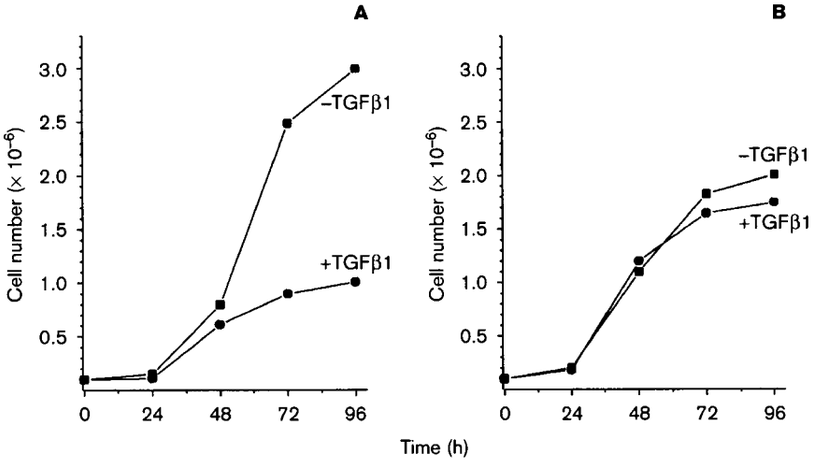

Figure 4. Effect of TGF-β1 (5 ng/ml) on the growth of L1210/S (A) and L1210/R (B) cells.

To be sure that both cisplatin and TGF-β1 influenced cell multiplication and not only [^{3}H]thymidine incorporation, we studied changes in the number of L1210/S and L1210/R cells grown in the presence of cisplatin (Figure 3) or TGF-β1 (Figure 4). Strong inhibition of the growth of L1210/S cells was observed in the presence of 0.1 μg/ml of cisplatin, while this drug concentration was ineffective for the inhibition of L1210/R cell growth (Figure 3). TGF-β1 was also an effective growth inhibitor of L1210/S cells and it did not affect the growth of L1210/R cells (Figure 4). Thus, both cisplatin and TGF-β1 clearly inhibited growth of L1210/S cells, and did not act in this manner when L1210/R cells were used.

Taking into account the transformed nature of murine L1210 leukemia cells, it was also reasonable to study the effect of cisplatin and TGF-β1 on these cells grown in a semisolid culture medium supplemented with 0.3% agar. The duration of this experiment was much longer (14 days) than that of the above described experiments and thus the long-term effects of different drugs could be estimated here. Figure 5 shows that cisplatin (1 μg/ml) practically abolished colony formation of L1210/S cells and was not effective at this concentration on L1210/R cells. TGF-β1 (5 ng/ml) inhibited the growth of L1210/S cells, while the L1210/R cells were resistant to the action of TGF-β1. The combined effect of both cisplatin and TGF-β1 was additive only for L1210/S cells.

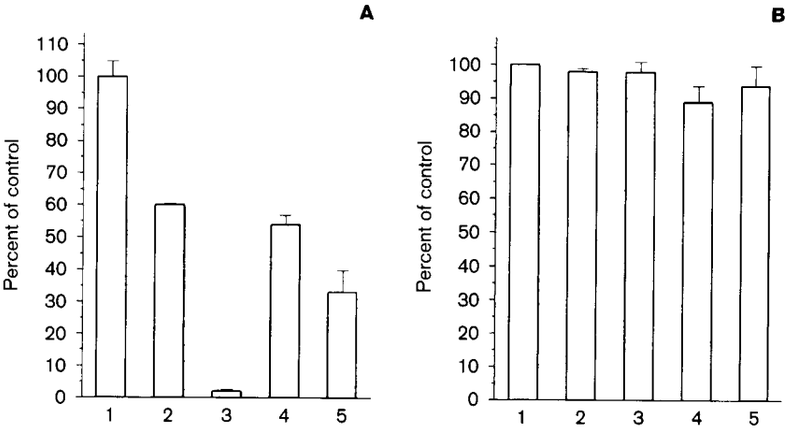

Figure 5. Effect of cisplatin and TGF-β1 on the colony formation by L1210/S (A) and L1210/R cells [1, control; 2, +cisplatin (0.1 μg/ml); 3, +cisplatin (1.0 μg/ml); 4, +TGF-β1 (5 ng/ml); 5, +cisplatin (0.1 μg/ml)+TGF-β1 (5 ng/ml)]. Ordinate: 100% for A was 667 colonies per 40 mm dish, for B was 95 colonies per 40 mm dish).

It is interesting to note that the morphology of colonies formed by L1210/R and L1210/S cells was considerably different. Compact spherical colonies were formed by L1210/S cells while disperse grape-like colonies appeared in the culture of L1210/R cells (Figure 6). The behavior of L1210/S and L1210/R cells in culture medium lacking 0.3% agar was also different. We found L1210/R cells to be attached to the bottom of the culture flask to a minor extent, while L1210/S cells grew as a typical suspension culture.

There is evidence that both cisplatin[14] and TGF-β1[8,10] can induce apoptosis in tumor cells. TGF-β1 has been shown to induce apoptosis in L1210 cells sensitive to the negative action of cisplatin.[10] Here we detected that TGF-β1 was ineffective in the induction of apoptosis in L1210/R cells (Figure 7). It should be noted that there was more apoptosis of L1210/S cells under TGF-β1 action when the cells were incubated in the absence of FCS. Such dependence on TGF-β1 was not observed in the case of cisplatin action, when apoptosis in L1210/S cells was observed in both the presence and absence of FCS in culture medium (Figure 7). One can see some development of apoptosis in L1210/S cells grown in FCS-free medium.

The method we used for DNA isolation[13] allows us to detect only low molecular weight apoptotic DNA which is released from the cell nucleus and then is extracted from the treated cells. That is why high molecular weight DNA could not be seen on our electrophoregrams (Figure 7).

Thus, we found a positive correlation between the resistance of murine leukemia L1210 cells to cisplatin action and their resistance to the growth inhibitory and apoptosis-inducing effects of TGF-β1.

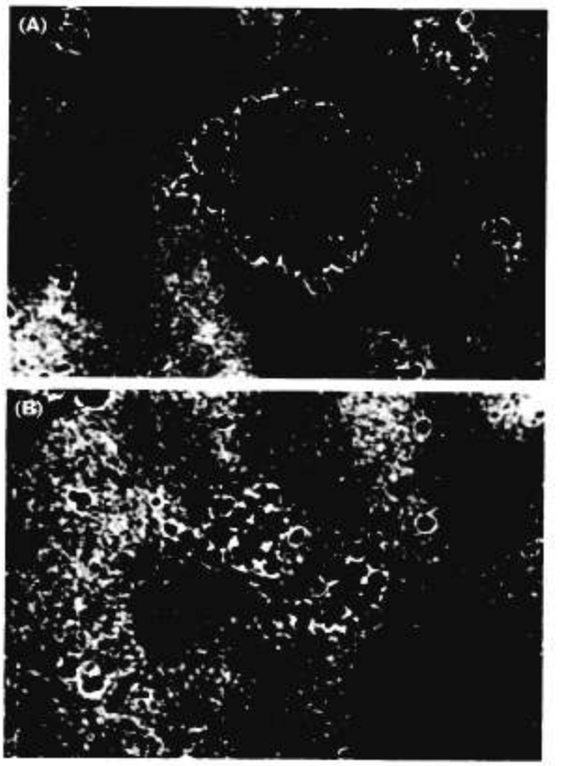

Figure 6. Typical colonies formed by L1210/S (A) and L1210/R (B) cells grown in semisolid culture medium supplemented with 0.3% agar (magnification × 60).

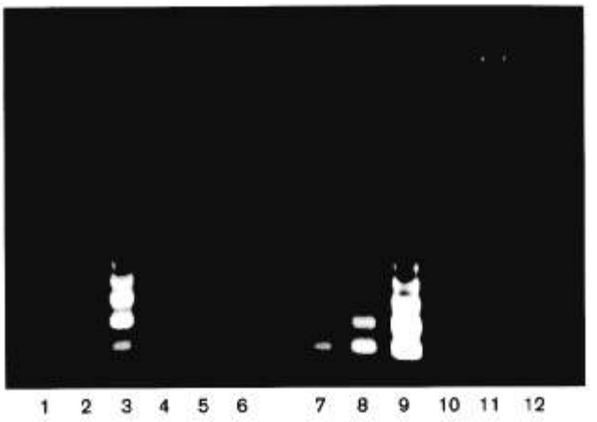

Figure 7. Effect of cisplatin and TGF-β1 on DNA fragmentation in L1210/S and L1210/R cells. DNA was prepared (see Materials and methods) from cells cultured for 24 h in serum-free medium before cisplatin (1 μg/ml) or TGF-β (5 ng/ml) addition for 48 h in the presence of 2% FCS (1–6) or in the absence of FCS (7–12). 1 and 7: Untreated L1210/S cells; 2 and 8: TGF-β1-treated L1210/S cells; 3 and 9: cisplatin-treated L1210/S cells; 4 and 10: untreated L1210/R cells; 5 and 11: TGF-β1-treated L1210/R cells; 6 and 12: cisplatin-treated L1210/R cells.

Discussion

Several mechanisms have been proposed for the appearance of cellular resistance to cisplatin in L1210 cells: (i) a reduced accumulation of the cisplatin,[15] (ii) an overexpression of specific 200 kDa membrane glycoprotein which is supposed to be analogous to P-glycoprotein and represent a non-functional gated channel,[16,17] (iii) change in cellular capacity to survive in the presence of platinum damage,[11] probably due to the enhanced capacity for DNA repair,[18] (iv) decreased expression of heat-shock protein 27,[19] and (v) elevated glutathione (GSH) level.[20]

In an attempt to understand further the mechanisms of cisplatin resistance in murine L1210 leukemia cells, we studied the sensitivity of parental and cisplatin-resistant lines of these cells to the action of TGF-β1. Earlier it was found that this cytokine inhibited growth and induced apoptosis in the cells of the parental line.[10,21]

Here we show that cisplatin-resistant L1210 cells were also refractory to the growth-inhibiting action of TGF-β1. To our knowledge this is the first example of positive correlation between the resistance of tumor cells to cisplatin action and their resistance to TGF-β1 inhibition. At present, the mechanism responsible for the appearance of such correlation is unknown, although it could be of great significance in view of the wide presence of TGF-β1 in mammalian tissues and organs, and the loss of sensitivity to TGF-β1 growth inhibition in various tumors, especially in the most malignant (metastatic) ones.[5,22]

It is possible that screening of TGF-β1 resistance in tumors could be useful for the detection of their cisplatin resistance. One may also speculate that the TGF-β1 regulatory system is somehow involved in the mechanisms of cisplatin antineoplastic action. We have found that the loss of sensitivity to cisplatin in L1210 cells is accompanied by the appearance of their resistance to TGF-β1-induced growth inhibition and of apoptosis.

Functional inactivation of TGF-β receptors or downstream signaling mediators was found to be one of the most common mechanisms responsible for cellular resistance to TGF-β-induced growth inhibition, although alternative mechanisms are also possible.[3,4] The regulatory mechanisms which could determine growth inhibition and apoptosis under TGF-β1 action on L1210 cells are poorly known. Ornitine decarboxylase, the key enzyme of the polyamine pathway, may have a role,[12] although the Ras/MAPK pathways and Smad signaling components that control TGF-β-mediated gene transcription were recently shown to be more probable candidates for such a role.[2–4] DNA repair is almost always increased in cisplatin-resistant cells while drug uptake, efflux, gluthatione level or metallothionein level may remain unchanged.[2] DNA repair appears to be activated first, so to achieve higher degrees of resistance, the cell may induce the above-mentioned additional mechanisms, including TGF-β1 resistance which we detected in this study.

It can be seen from our results that the apoptosis-inducing effect of TGF-β1 for L1210 parental cells sensitive to cisplatin action is expressed better when these cells were incubated in FCS-free culture medium than when they were treated with TGF-β1 in the presence of FCS. This dependence of apoptosis development upon FCS constituents was not observed in the case of cisplatin action which showed its apoptosis-inducing effect both in the presence and absence of FCS. This suggests that additional factors available in blood serum can modulate the apoptosis-inducing action of TGF-β1.

In conclusion, our results indicate a possible interrelation between the mechanisms of cellular resistance to cisplatin and to TGF-β1. This result could be of great importance for the development of new approaches to diminish the resistance of tumor cells to cisplatin action.

References

1. Gately DP, Howell SB. Cellular accumulation of the anticancer agent cisplatin: a review. *Br J Cancer* 1993; **67**: 1171-6.
2. Chu G. Cellular responses to cisplatin. *Cancer* 1994; **269**: 787-90.
3. Massague J. TGF-β signal transduction. *Annu Rev Biochem* 1998; **67**: 753-91.
4. Derynk R, Feng XH. TGF-β receptor signaling. *Biochim Biophys Acta* 1997; **1333**: F105-50.
5. Hurta RAR, Samual SK, Greenberg AH, Wroght JA. Early induction of ribonucleotide reductase gene expression by transforming growth factor β1 in malignant Ha-*ras* transformed cell lines. *J Biol Chem* 1991; **266**: 24097-100.
6. de Cotean JF, Kuans PI, Yankelev H, *et al.* Loss of functional cell surface transforming growth factor beta (TGF-beta) type 1 receptor correlates with insensitivity to TGF-beta in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 1997; **94**: 5877-81.
7. Raynal S, Nocentini S, Croisy A, Lawrence DA, Julien P. Transforming growth factor-beta 1 enhances the lethal effects of DNA-damaging agents in a human lung-cancer cell line. *Int J Cancer* 1997; **72**: 356-61.
8. Maccarone M, Nieuwenkuizen WE, Dulens HF, *et al.* Membrane modification in human eruthroleukemia K562 cells during induction of programmed cell death by transforming growth factor beta 1 or cisplatin. *Eur J Biochem* 1996; **241**: 297-302.
9. McCornack SE, Bruskin AM, Borzillo GV. A p53 growth arrest protects fibroblasts from anticancer agents. *BioSci* 1997; **1**: A37-45.
10. Motyl T, Kasterka M, Grzelkowska K, *et al.* Phorbol ester (12-*O*-tetradecanoyl phorbol 13-acetate) prevents ornithine decarboxylase inhibition and apoptosis in L1210 leukemic cells exposed to TGF-beta 1. *Int J Biochem Cell Biol* 1996; **28**: 1327-35.
11. Waud WR. Differential uptake of *cis*-diaminedichloroplatinum (II) by sensitive and resistant murine L1210 leukemia cells. *Cancer Res* 1987; **47**: 6549-55.
12. Rizzino A. Soft agar growth assays for transforming growth factors and mitogenic peptides. In: Barnes D, Sirbasku DA, eds. *Methods in enzymology*. New York: Academic Press 1987: 341-52.
13. Herrmann M, Lorenz HM, Voll R, Grunke M, Woith W, Kalden JR. A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acid Res* 1994; **22**: 5506-7.
14. Segal-Bendirdjian E, Jacquemin-Sablon A. Cisplatin resistance in a murine leukemia cell line is associated with a defective apoptotic process. *Exp Cell Res* 1995; **218**: 201-12.
15. Kraker AJ, Moore CW. Accumulation of *cis*-diaminedichloroplatinum (II) and platinum. *Cancer Res* 1988; **48**: 9-13.
16. Kawai K, Kamavatani N, Georges E, Ling V. Identification of a membrane glycoprotein overexpressed in murine lymphoma sublines resistant to *cis*-diaminedichloroplatinum (II). *J Biol Chem* 1990; **265**: 13137-42.
17. Stasyk TV, Antonyuk VA, Yakymovych MYa, *et al.* A comparative study of cell surface glycosyl determinants in sensitive and resistant to cisplatin L1210 murine leukemia cells. *Exp Oncol* 1998; **20**: 204-9.
18. Sheibani N, Jennerwein MM, Eastman A. DNA repair in cells sensitive and resistant to *cis*-diaminedichloroplatinum(II); host cell reactivation of damaged plasmid DNA. *Biochemistry* 1989; **28**: 3120-5.
19. Hettinga JVE, Lemstra W, Meijer C, *et al.* Mechanism of hyperthermic potentiation of cisplatin action in cisplatin-sensitive and -resistant human tumour cells. *Br J Cancer* 1997; **75**: 1735-43.
20. Hronas RA, Andrew PA, Murphy MP, Burns CP. Glutathione depletion reverses cisplatin resistance in murine L1210 leukemia cells. *Cancer Lett* 1987; **34**: 9-15.
21. Haak-Frendscho M, Wynn TA, Czuprynski CJ, Paulnock D. Transforming growth factor-beta 1 inhibits activation of macrophage cell line RAW 264.7 for cell killing. *Clin Exp Immunol* 1990; **82**: 404-10.
22. Barrack ER. TGF beta in prostate cancer: a growth inhibitor that can enhance tumorigenecity. *Prostate* 1997; **31**: 61-70.

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