ONCOLOGY

Combined Effect of Cisplatin and Lymphokine-Activated Killer Cells on A549 Cells of Non-Small Cell Lung Cancer

O. S. Zhukova, G. K. Gerasimova, I. Zh. Shubina, and M. V. Kiselevskii

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We studied the ability of lymphokine-activated killer cells to lyse A549 human non-small cell lung cancer cells after preincubation with cisplatin. Lymphokine-activated killer cells obtained after incubation of human blood lymphocytes with interleukin-2 were characterized by high expression of natural killer cell antigens and activation molecules. Lymphokine-activated killer cells produced potent cytotoxic effect on intact A549 cells and lysed tumor cells survived after treatment with cisplatin in concentrations of IC_{50} and IC_{30}. Cisplatin in noncytotoxic concentrations did not increase lytic activity of lymphokine-activated killer cells.

Key Words: *lymphokine-activated killer cells; non-small cell lung cancer; cisplatin; cytotoxicity*

Non-small cell lung cancer (NSCLC) is one of the most common cancer diseases. When the diagnosis is made, most patients have disseminated disease and should be subjected to nonsurgical therapy. Chemotherapy for NSCLC usually includes treatment with platinum derivatives (cisplatin, DDP) [1,8,12]. The clinical effectiveness of chemotherapy for NSCLC is 25-40% [7,8,12]. The methods of combined chemotherapy and biotherapy for NSCLC are developed to increase the effectiveness of conservative treatment [9].

Chemoresistant cells are sensitive to cytotoxic lymphocytes, natural killer cells, and lymphokine-activated killer cells (LAKC) [2-4,6,11,15]. They produce a potent lytic effect on explants of soft tissue sarcoma and human epithelial tumors that are resistant to cytostatics doxorubicin, cyclophosphamide, methotrexate, vincristine, and carboplatin [2]. Several authors reported that antitumor cytostatics in noncytotoxic doses increase lytic activity of LAKC to several lines of tumor cells [4,7,10]. It remains unknown whether LAKC can lyse survived tumor cells after chemotherapy.

Here we studied the effectiveness of combined treatment of human NSCLC cells with DDP and LAKC.

MATERIALS AND METHODS

Experiments were performed on human tumor cells, A549 NSCLC cells and K562 erythroblastic leukemia cells (American Type Culture Collection).

The cells were grown on RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

Light microscopy and photographing of cells were performed using an AxioVision 4 system (Zeiss).

Blood mononuclear leukocytes were isolated from peripheral blood samples obtained from 10 healthy donors and stabilized with 25 U/ml heparin. The cells were centrifuged at 400*g* for 30 min using a one-step Ficoll gradient (1.077 g/cm^3, Pan-

N. N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** kisele@inbox.ru. M. V. Kiselevskii

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Eko). Lymphoid cells forming the interphase ring were collected with a pipette and washed 3 times in 10-fold volume medium 199 with centrifugation at 200*g*.

Peripheral blood mononuclear leukocytes (10^6 cells/ml) were resuspended in RPMI-1640 medium with 10% FBS. Interleukin-2 (IL-2, Proleukine, Chiron) in a concentration of 1000 U/ml was added. The cells were incubated at 37°C and 5% CO_2 for 2 days.

The cells were put in 96-well flat-bottom microplates (Costar, 10^4 cells per well) and incubated for 24 h. DDP (Teva) was added to wells. The final concentrations were 10^{-5}-5×10^{-5} M. After 1 day, these cells were washed from the cytostatic, mixed with LAKC, and incubated for 24 h. In the control series, LAKC or DDP in specified concentrations were added to cells. Functional activity of LAKC was tested on K562 cells sensitive to natural killer cells. The effector/target cell ratio was 1:5.

The cytotoxic effect was evaluated by the MTT test. This test is based on reduction of colorless dye to DMSO-soluble blue formazan crystals by dehydrogenases in living cell.

Optical density of DMSO solutions was measured on a Multiscan MS spectrophotometer (Labsystems) at 540 nm.

The inhibition of cell growth was calculated by the formula: $(1\text{-}N_T/N_C)\times100\%$, where N_T is optical density of treated samples; and N_C is optical density of control samples. The error of measurements did not exceed 5%.

Expression of surface markers on effector cells was studied using monoclonal antibodies against the corresponding antigens (Caltag Laboratories). Flow cytofluorometry was performed on a FACScan flow cytometer (Becton Dickinson). We studied the expression of differentiation antigens (D3, CD4, CD8, and CD16), activation antigens (CD25, CD38, and HLA-DR), and adhesion molecules (CD57 and CD58). The gate of cell population was set from the combination of forward and side scatter and cell size. We counted 10,000 events per gate.

The results were analyzed by Winmdi 2.8 software.

RESULTS

After incubation with IL-2, mononuclear leukocytes were characterized by high expression of natural killer cell antigens (CD16 and CD56), adhesion molecules (CD58), and activation antigens (CD25 and CD38). Cytotoxicity of LAKC to natural killer cell-sensitive K562 cells increased from 54.0±3.7 to 78.0±5.9% ($p<0.05$). Hence, the cells obtained after coincubation with IL-2 can be attributed to the category of LAKC by immunophenotypic and functional characteristics.

Intact A549 cells had a polygonal shape and vacuolated cytoplasm and formed a dense monolayer. Insoluble violet formazan crystals were seen in the cytoplasm after vital staining with MTT reagent. These data illustrate viability of cells (Fig. 1, *a*, *b*).

Formazan crystals were identified in the area of LAKC clusters and cytoplasm of individual survived tumor cells after exposure of intact A549 cells to the cytotoxic effect of LAKC (Fig. 1, *c*, *d*). Lytic activity of LAKC was 69% (Table 1).

Incubation of A549 cells with DDP in a concentration of 5×10^{-5} M was followed by lysis of 48% cells, which corresponded to IC_{50} (concentration of the preparation inhibiting growth of 50% cells; Fig. 1, *e*, *f*). After 24-h incubation of LAKC with survived tumor cells, cell viability in samples did not exceed 10%. Under these conditions, formazan crystals stained only LAKC clusters (Fig. 1, *g*, *h*).

Similar results were obtained after combined treatment with DDP in a concentration of $2.5*10^{-5}$ M (IC_{30}) and LAKC. DDP in a concentration of below IC_{30} produced minor cytotoxic effect. Cell death did no exceed 4-10% (Table 1). Further incubation of tumor cells with LAKC also caused death of tumor cells, which was comparable with that observed after exposure of intact tumor cells to the cytotoxic effect of LAKC (Table 1).

We conclude that LAKC can lyse tumor cells survived after treatment with DDP in concentrations of IC_{50} and IC_{30}. Preincubation of A549 cells with DDP in lower (noncytotoxic) concentrations

TABLE 1. Cytotoxic Effect of DDP, LAKC, and LAKC+DDP on A549 Tumor Cells

Drug	DDP concentration, M			
	1×10^{-5}	1.25×10^{-5}	2.5×10^{-5}	5×10^{-5}
DDP	4.0±2.1	10.5±7.6	30.6±3.9	48.1±5.2
LAKC+DDP	69.0±4.7	72.2±7.5	87.3±6.3	90.6±9.2

Note. Inhibition of cell growth after addition of LAKC alone was 69.3±3.2%.

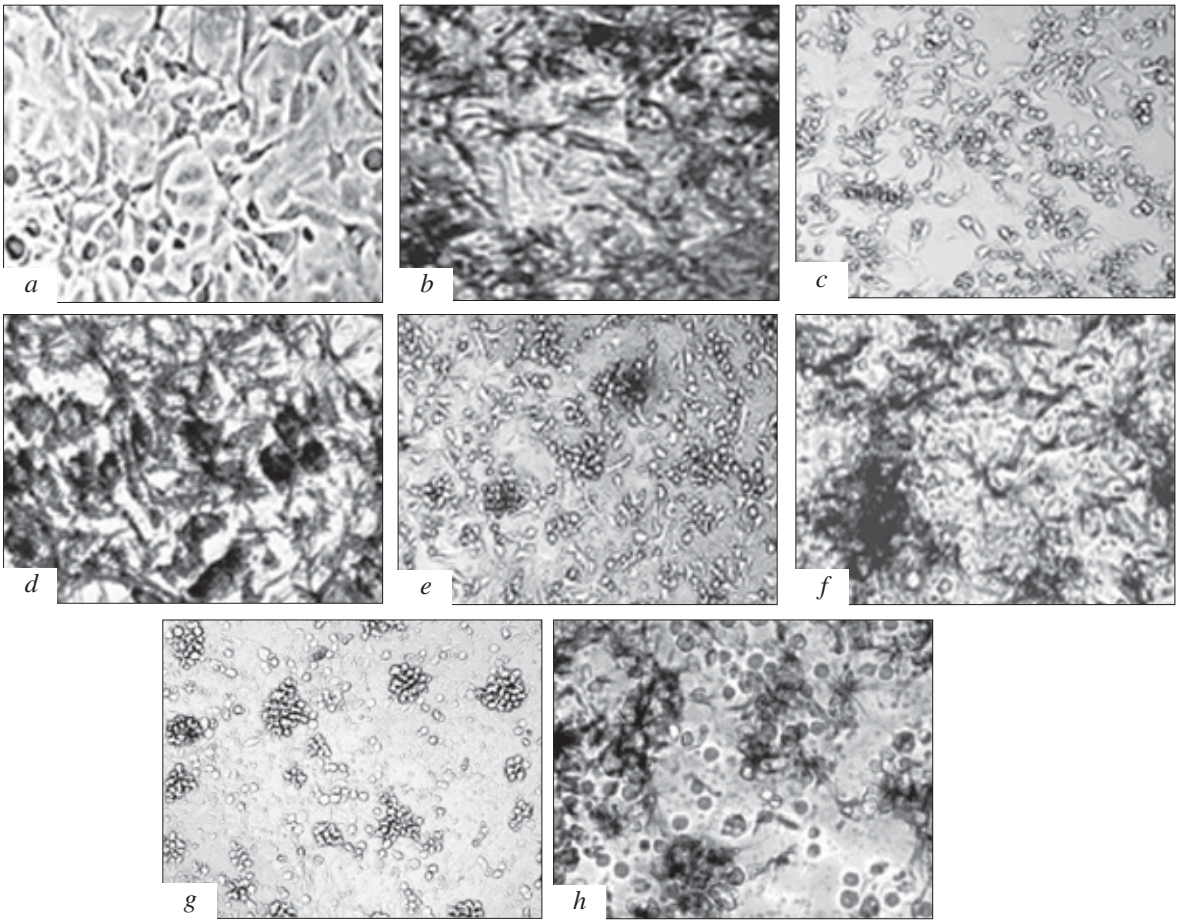

Fig. 1. Cytotoxic activity of DDP, LAKC, and LAKC+DDP to A549 NSCLC cells (×400). Intact A549 cells (*a*); A549 cells after addition of a vital staining agent MTT (*b*); A549 cells after incubation with DDP (IC_{50}, *c*); MTT-stained A549 cells after incubation with DDP (IC_{50}, *d*); A549 cells after incubation with LAKC (*e*); MTT-stained A549 cells and LAKC (*f*); individual A549 cells after incubation with DDP in IC_{50} and LAKC (*g*); individual MTT-stained A549 cells after incubation with DDP in IC_{50} and LAKC (*h*).

did not potentiate the lytic effect of LAKC. Our results contradict published data on the increase in sensitivity of tumor cells to LAKC after treatment with low doses of DDP [2,10]. This discrepancy is probably related to different experimental conditions. In these studies, tumor cells were incubated in the presence of low concentrations of DDP and then treated with LAKC. The cytostatic was not removed from the nutrient medium. Previous experiments showed that the cytostatic in low concentrations can increase cytotoxicity of lymphocytes due to inhibition of suppressor subpopulation [7].

DDP belongs to a group of alkylating chemotherapeutics inhibiting DNA replication. The sensitivity of tumor cells to DDP is maximum in G_1 phase and minimum in S phase of the cell cycle [3,5,14]. The cytotoxic effect of DDP correlates with DNA damage, which depends on its dose. This drug lengthens S phase, but inhibits the cells in G_2 phase. Phases of the cell cycle in cells survived after exposure to DDP are insensitive to this drug [13,14]. The cytotoxic effect of LAKC probably does not depend on phase of the cell cycle, which contributes to lytic activity relative to DDP-resistant cells.

The concept of combined chemotherapy and immunotherapy to increase the effectiveness of antitumor treatment was extensively developed in recent years [4,9,11]. Combined treatment with platinum chemotherapeutics and LAKC is one of the promising approaches to the therapy for NSCLC. Our results illustrate the rationality of combined treatment with chemotherapeutics and LAKC, which prevents the development of tumor drug resistance without increasing the dose of the cytostatic.

REFERENCES

1. S. L. Gutorov, *Ros. Med. Zh.*, **14**, 1032-1035 (2006).
2. H. D. Bear, *Cancer Res.*, **46**, No. 4, Pt. 1, 1805-1812 (1986).
3. N. M. Berezhnaya, U. D. Vinnichuk, V. F. Konovalenko, *et al.*, *Exp. Oncol.*, **27**, No. 4, 303-307 (2005).

4. R. Chen, L. Pan, and S. Zhou, *Zhonghua Fu Chan Ke Za Zhi*., **34**, No. 3, 172-174 (1999).
5. K. L. Donaldson, G. L. Goolsby, and A. F. Wahl, *Int. J. Cancer*, **57**, No. 6, 847-855 (1994).
6. S. C. Gautan, N. F. Chikkala, I. Lewis, *et al*., *Anticancer Res*., **12**, No. 3, 921-925 (1992).
7. T. H. Inge, S. K. Hoover, J. L. Frank, *et al*., *Cancer Immunol. Immunother*., **35**, No. 2, 119-126 (1992).
8. K. Kelly, J. Crowley, and P. Bunn, *J. Clin. Oncol*., **19**, No. 3, 3210-3218 (2001).
9. H. Kimura and Y. Yamaguchi, *Cancer*, **80**, No. 1, 9-25 (1997).
10. I. Matsuzaki, H. Suzuki, M. Kitamura, *et al*., *Oncology*, **59**, No. 4, 336-343 (2000).
11. A. Ozkan, A. Ayhan, and K. Fiskin, *Cell. Biol. Toxicol*., **20**, No. 5, 261-271 (2004).
12. J. H. Schiller, D. Harrington, and C. P. Belani, *N. Engl. J. Med*., **346**, No. 2, 92-98 (2002).
13. M. A. Shah and G. K. Schwartz, *Clin. Cancer Res*., **7**, No. 8, 2168-2181 (2001).
14. C. M. Sorenson and A. Eastman, *Cancer Res*., **48**, No. 16, 4484-4488 (1988).
15. Y. J. Zheng, J. Zhang, S. M. Wang, and J. R. Zhang, *Di Yi Yi Da Xue Xue Bao*, **24**, 392-396 (2004).