In Vitro Exposure of NK-92 Cells to Serum from Patients with Non-small Cell Lung Cancer Impairs Their Cytotoxicity

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Abstract. *Aim: To investigate the effect of serum from patients with non-small cell lung cancer (NSCLC) on in vitro cytotoxicity of the clonal cell line NK-92. Materials and Methods: Twenty-six patients with NSCLC were included in this study. NK-92 cells were incubated in medium supplemented with 25% of each patient's serum (before and after chemotherapy) for 24 h, then washed and tested for cytotoxicity against NK-sensitive K562 targets. Results: The cytotoxicity of NK-92 cells exposed to serum from patients with NSCLC before chemotherapy initiation was significantly reduced compared to that upon incubated with serum from healthy individuals ($p<0.001$). NK-92 cytotoxicity was further reduced upon exposure to patient's serum after the first and third chemotherapy cycles ($p<0.001$). Conclusion: The results of our in vitro study suggest that serum of patients with NSCLC may exert per se an inhibitory effect on the cytotoxicity of NK-92 cells and this negative regulation may be enhanced with chemotherapy.*

Conventional chemotherapy agents have been proven to be largely ineffective in treating several solid malignancies, as they only affect rapidly dividing non-stem cancer cells and their therapeutic benefit is limited by significant toxicity and frequent development of drug resistance (1, 2). Consequently, a broad range of emerging treatment strategies, including adoptive cellular immunotherapy, are being intensively investigated for potential use in cancer therapeutics, either as an alternative to or in combination with standard chemotherapy. Many of these research efforts, including some ongoing clinical trials, aim to enhance or compromise the antitumor-reactive cytotoxicity of immune effectors, principally of cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells (2-6).

NK cells are a distinct lymphocyte subset with a crucial role in first-line immune defense against virus-infected and malignant cells (7). In contrast to CTLs which require activation by peptide fragments bound to major histocompatibility complex (MHC) molecules, non-MHC-restricted NK cells can rapidly and spontaneously kill their targets without prior antigen sensitization (4, 8). The final outcome of their interaction with target cells is controlled by a delicate balance between activating and inhibitory signals transmitted by NK cell surface receptors, leading to target cell lysis or inhibition of lysis, respectively (8). This process is mediated by several cytokines, including interleukins (IL)2, 12, 15, 18 and 21, reported to significantly enhance the cytotoxic activity of NK cells (4, 9).

According to previous experimental data, chemotherapy regimens may reduce the number of NK cells and compromise their cytotoxic activity (10, 11). Therefore, concerns have been raised with regard to the potential interactions between conventional cytotoxic agents and NK cell-based immunotherapy (10, 12).

The aim of the present study was to investigate the potential effect of chemotherapy on the *in vitro* cytotoxicity of the clonal cell line NK-92. For this, NK-92 cells were incubated with serum obtained from patients with non-small cell lung cancer (NSCLC), before and after administration of chemotherapy, and the recorded levels of cytotoxicity were correlated to the treatment regimes.

Materials and Methods

Patients. Study participants were admitted to the Oncology Unit of the Sotiria General Hospital, Athens, Greece. The protocol was approved by the Institutional Review Board of the University of Athens (approval number: 201/2004) and all patients provided a written informed consent.

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Key Words: Non-small cell lung cancer, NK cells, NK-92, cellular immunotherapy.

Table I. *Clinicopathological characteristics of patients with non-small cell lung cancer.*

Variable	Value
Mean age (SD), years; range	63.4 (9.2); 45-77
Gender, N (%)	
Male	22 (84.6)
Female	4 (15.4)
Smoking status, N (%)	
Smokers	23 (88.5)
Non smokers	3 (11.5)
Tumor grade, N (%)	
Low	9 (34.6)
Moderate	15 (57.7)
High	2 (7.7)
Disease stage, N (%)	
IIIA	5 (19.2)
IIIB	4 (15.4)
IV	17 (65.4)
Radiotherapy, N (%)	
Yes	3 (11.5)
No	23 (88.5)
Chemotherapy, N (%)	
Group A (docetaxel/gemcitabine)	13 (50.0)
Group B (docetaxel)	8 (30.8)
Group C (paclitaxel/gemcitabine)	5 (19.2)

Table II. *Descriptive data and analysis of variance (ANOVA) results for NK-92 cell cytotoxicity prior to chemotherapy (T1), and at one (T2) and three (T3) months after the first chemotherapy cycle in patients with non-small cell lung cancer (NSCLC) and healthy individuals.*

ANOVA	Group	Time-point	Mean (SD) cytotoxicity (%)	*p*-Value
One way-ANOVA	NK-92‡		69.33 (5.03)	<0.001
	Healthy individuals (n=3)	T1	54.67 (2.51)	
	Patients with NSCLC (n=26)	T1	40.81 (9.49)	
Within subjects repeated measures (ANOVA)	Patients with NSCLC (n=26)	T1 T2 T3	40.81 (9.49) 34.82 (11.02) 24.60 (10.23)	<0.001
Within and between-subjects repeated measures (ANOVA)	Group A (n=13)	T1 T2 T3	41.97 (9.18) 34.72 (12.17) 21.71 (10.88)	<0.001
	Group B (n=8)	T1 T2 T3	42.79 (9.43) 36.27 (9.14) 25.00 (10.86)	<0.001
	Group C (n=5)	T1 T2 T3	34.60 (9.67) 27.04 (10.56) 21.20 (7.78)	<0.001

‡NK-92 Cells cultured in complete alpha minimum essential medium; group A: patients with NSCLC treated with docetaxel plus gemcitabine; group B: patients with NSCLC treated with docetaxel; group C: patients with NSCLC treated with paclitaxel plus gemcitabine.

Patients recruited in this study had histologically-confirmed stage III/IV NSCLC and satisfactory performance status [<2 by Eastern Cooperative Oncology Group (ECOG) criteria], and received three consecutive cycles of chemotherapy during the study period. The chemotherapy regimens administered were 100 mg/m^2 docetaxel plus 1250 mg/m^2 gemcitabine (group A; 13 patients); 100 mg/m^2 docetaxel (group B; 8 patients); and 130 mg/m^2 paclitaxel plus 1,250 mg/m^2 gemcitabine (group C; 5 patients). Three patients additionally received adjuvant radiotherapy for brain metastases.

Serum isolation. Non-heparinized peripheral blood samples (10 ml) were aseptically collected at the following time-points: prior to chemotherapy, on the day of the first chemotherapy cycle (T1; basal); one month after administration of the first chemotherapy cycle, on the day of the second chemotherapy cycle before administration of chemotherapy (T2); and three months after administration of the first chemotherapy cycle (T3). Following centrifugation at 830 xg 2,500 rpm for 15 min at room temperature, serum was removed, aliquoted and stored at –80˚C. Serum samples from three healthy individuals were used as controls.

Cell lines. The NK-92 cell line [American Type Culture Collection (ATCC), Rockville, MD, USA] was expanded in alpha minimum essential medium (α-MEM; Gibco-BRL, Grand Island, NY, USA), supplemented with 12.5% heat-inactivated fetal calf serum (FCS; Gibco-BRL), 12.5% heat-inactivated horse serum (Gibco-BRL), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.2 mM inositol, 2 μM folic acid, 0.05 mM 2-mercaptoethanol (all from Sigma Chemical Co., St. Louis, MO, USA), and anti-microbial agents (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL) in the presence of 200 IU/ml human recombinant IL2 (Proleukin, Cetus Corp., Los Angeles, CA, USA) (thereafter referred to as complete α-MEM), at 37˚C with 5% CO_2 (13).

The NK-sensitive erythroleukemia cell line K562 (ATCC) was similarly propagated in RPMI-1640 (Gibco-BRL), supplemented with 10% FCS, 2 mM L-glutamine and the aforementioned antimicrobial agents (thereafter referred to as complete RPMI) (14).

NK-92 cell exposure to serum from patients with NSCLC. NK-92 cells were expanded at logarithmic phase and further incubated for 24 h in complete α-MEM supplemented with 25% of each patient's serum. NK-92 cells were washed with Hank's balanced salt solution (Gibco-BRL) and immediately used as effectors in a standard cytotoxicity assay.

Cytotoxicity assay. K562 cells were intracellularly labeled with sodium chromate and washed, as previously described (14). They were further resuspended in complete RPMI (1×10^5/ml) and used as targets. Serum-exposed effector cells (1×10^6/ml in complete α-MEM; 100 μl/well) were plated in 96-well U-bottom plates

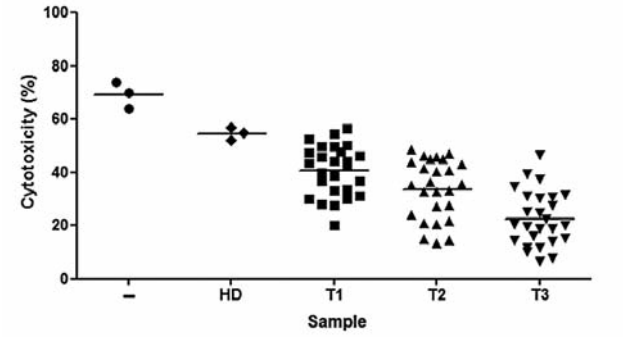

Figure 1. *Cytotoxicity of NK-92 cells exposed to serum of healthy individuals and patients with non-small cell lung cancer (NSCLC). NK-92 cells were incubated in complete α-MEM (–), with serum from healthy donors (HD), or with serum from patients with NSCLC prior to chemotherapy (T1), one month after administration of the first chemotherapy cycle (T2), and three months after administration of the first chemotherapy cycle (T3). K562 cells were used as targets and in all experiments, the effector: target ratio was 10:1. Each data point represents the cytotoxicity acquired per single individual (healthy donors n=3; patients with NSCLC n=26); bars indicate mean values of data from each group. *p<0.001, compared with (–).*

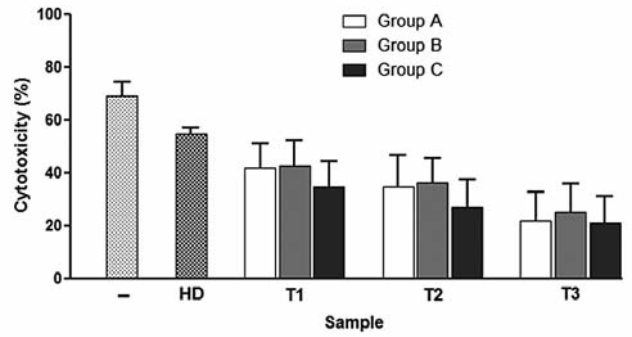

Figure 2. *Cytotoxicity of NK-92 cells exposed to serum of patients with non-small cell lung cancer (NSCLC) administered docetaxel plus gemcitabine (group A), docetaxel (group B) and paclitaxel plus gemcitabine (group C). NK-92 cells were incubated in complete α-MEM (–), with serum from healthy donors (HD), or with serum from patients with NSCLC prior to chemotherapy (T1), one month after administration of the first chemotherapy cycle (T2), and three months after administration of the first chemotherapy cycle (T3). K562 cells were used as targets and in all experiments, the effector: target ratio was 10:1. Bars represent means±SD.*

(Greiner Bio-one, Kirchheim, Germany) and target cells were added (100 μl/well) to obtain a final effector:target cell ratio of 10:1. After co-incubation for 4 h, at 37˚C with 5% CO_2, 100 μl of supernatant was removed from each well. Isotope release was determined in a γ-counter (1275 Mini-gamma LKB; Wallac, Turku, Finland) and expressed as counts per minute (cpm). Maximal and spontaneous cpm were determined in wells where T-cells were incubated with 3 N HCl and in complete RPMI, respectively. All assays were carried out in triplicate. Specific T-cell lysis exerted by serum-exposed NK-92 effectors was calculated according to the formula: cytotoxicity (%)=[(experimental cpm–spontaneous cpm)/(maximal cpm–spontaneous cpm)]×100. Mean cytotoxicity values were used for statistical analysis.

Statistical analysis. All continuous data were screened for normality using the Shapiro-Wilk one-sample test, as the sample size was smaller than 50. The mean and standard deviation (SD) were obtained for all continuous data. The cytotoxicity values calculated at the three time-points (T1, T2 and T3) were compared within and between groups using one-way analysis of variance (ANOVA) and repeated measures ANOVA (RM-ANOVA). Sidak *post-hoc* testing was undertaken to establish the nature of statistically significant interactions. The alpha value was set at $p<0.05$ and analyses were conducted using SPSS statistical software (IBM Corp., New York, NY, USA).

Results

A total of 26 patients with NSCLC (22 males and four females; mean age=63.4 years; range=45-77 years) were enrolled in the study. Demographics and clinicopathological characteristics of patients at study initiation are summarized in Table I.

The mean cytotoxicity of NK-92 cells cultured in complete α-MEM was 69.33% (SD=5.03%). When the same cells were incubated for 24 h in complete α-MEM supplemented with 25% serum of healthy individuals, their cytotoxicity decreased to 54.67% (SD=2.51%; Figure 1 and Table II). In preliminary experiments, supplementation of complete α-MEM with an increased percentage of serum (50% and 75%) resulted in substantial reduction of NK-92 cell viability (data not shown). After incubation of NK-92 cells with sera from patients with NSCLC prior chemotherapy administration (T1), the mean NK-92 cell cytotoxicity further decreased to 40.81% (SD=9.49%). One month following administration of the first chemotherapeutic cycle (T2), the ability of NK-92 cells to lyse K562 targets was additionally reduced to 34.82% (SD=11.02) and at three months (T3), this decrease was even more prominent (24.60%; SD=10.23). However, mean cytotoxicity values did not significantly differ between patient groups treated with different chemotherapy schedules (21.71±10.88%, 25±10.86% and 21.20±7.28% for groups A, B and C, respectively; Figure 2 and Table II).

The results of descriptive statistics and ANOVA showing the significance of differences in NK-92 cell cytotoxicity values between groups and time-points are presented in Table II. A statistically significant difference in mean basal (T1) cytotoxicity was found between the total patient population, healthy controls and NK-92 cells incubated in complete α-MEM ($p<0.001$, by one-way ANOVA). A statistically significant reduction of cytotoxicity was also observed between basal (T1) and subsequent time-points (T2 and T3)

for the overall patient population ($p<0.001$, by within-patients RM-ANOVA). The *post-hoc* test revealed a statistically significant reduction of cytotoxicity between time-points T1 versus (*vs.*) T2, T2 *vs.* T3 and T1 *vs.* T3 in the overall patient population (in all cases, $p<0.001$). Further comparison of cytotoxicity values within and between groups using RM-ANOVA followed by *post-hoc* test, revealed a statistically significant reduction of cytotoxicity between basal (T1) and subsequent time-points (T2 and T3) in each treatment group (in all cases, $p<0.001$). In contrast, no statistical correlation was found between the reduction of cytotoxicity and the type of chemotherapeutic regimen administered ($p=0.378$).

Discussion

Autologous or allogeneic NK cells have been used in NK-based adoptive cellular immunotherapy trials for treating various malignancies, but despite some promising results, most of these studies failed to yield consistent and substantial clinical benefit (3, 4, 15-20). Several reasons may account for this, including the *in vivo* inhibition of NK cell activity by MHC class I molecules expressed on cancer cells, impaired functionality of NK cells in patients with advanced cancer due to the disease/ therapeutic interventions, as well as technical difficulties associated with the isolation and large-scale *ex vivo* expansion of cancer patient-derived NK cells (2, 21, 22).

To overcome these limitations, human NK cell lines have been established from patients with hematological malignancies (21, 22). Among them, the highly cytotoxic cell line NK-92, established from a male patient with non-Hodgkin's lymphoma, represents a suitable candidate for clinical use in cancer immunotherapy (22). Although the *in vitro* lytic ability of NK-92 cells is IL2-dependent (23, 24), it is still retained *in vivo*, as shown in tumor models in severe combined immunodeficient mice (25). The high cytotoxicity of NK-92 cells, in combination with their easily controlled growth in culture, and their rapid and adequate expansion under good manufacturing practice conditions (13), supported their use in phase I clinical trials (2, 26).

In recent years, accumulating evidence suggests that antitumor immunity is of crucial importance for the achievement of optimal clinical responses towards conventional cytotoxic chemotherapy, and, conversely, chemotherapy may affect antitumor immune responses, either by promoting or by impeding various steps in this process (1). This complex and largely unexplored interplay between chemotherapy and immune defense mechanisms may also explain the frequent failure of conventional cytotoxic or immunotherapeutic agents to generate clinically significant and long-lasting treatment results when either of them is used as monotherapy in patients with cancer.

As shown by previous experimental and clinical studies, administration of chemotherapy in combination with immunotherapy enhances antitumor responsiveness in various forms of cancer, including NSCLC (12, 27). These findings are consistent with recent data suggesting that lung cancer may be susceptible to immunotherapy, as it is not as poorly immunogenic as previously believed (28-30). In mouse models, NK cells have been shown to participate in immune responses to lung cancer by protecting against the development of metastases (31). However, studies in patients with NSCLC have shown that their NK cells are functionally deficient and demonstrate reduced infiltration into lung tumors, suggesting that NK cell cytotoxicity in these patients is impaired (28, 32).

The cytotoxicity of NK cells is reportedly dependent on microtubule integrity, and the direct inhibitory effect of topoisomerase inhibitors and antimicrotubule drugs on some NK cell functions has been already demonstrated (10, 11, 33). According to the report of Markasz *et al.*, treatment of NK cells with certain cytotoxic drugs (including the antimicrotubule agents paclitaxel and docetaxel) may inhibit NK cell-mediated killing without affecting their viability (10). Nonetheless, despite these intriguing observations, relatively little is known regarding the cumulative and long-term effect of chemotherapy on the cytotoxicity of NK cells.

In the present study, we investigated the effect of peripheral blood serum from patients with advanced NSCLC on the *in vitro* cytotoxic activity of the clonal cell line NK-92 before chemotherapy administration, as well as at defined time-points during the course of three consecutive cycles of chemotherapy. Our results show that the cytotoxicity of NK-92 cells exposed to serum from patients with NSCLC before initiation of chemotherapy (basal) was significantly reduced compared to the cytotoxicity of NK-92 cells incubated with serum from healthy individuals. Furthermore, a consistent decrease in NK-92 cell cytotoxicity was observed upon incubation with sera obtained at subsequent time-points (*i.e.* one and three months after chemotherapy administration). These results, albeit limited by the relatively small number of cases studied, suggest that the serum of patients with NSCLC potentially contains mediators that down-regulate NK-92 cell cytotoxicity and that administration of chemotherapy further diminishes NK cell lytic activity.

In conclusion, the results of our preliminary *in vitro* study indicate that the cytotoxic activity of NK-92 cells may be down-regulated by factors included in the serum of patients with NSCLC, and this impairment is further enhanced upon treatment with anti-microtubule agents, such as paclitaxel or docetaxel. Identification of NK cell-inhibitory molecules contained in serum from patients with cancer and *in vivo* studies are warranted to explore the potential implication of our findings on the efficacy of combinatorial therapeutic strategies involving chemotherapy agents in conjunction with NK cell-based immunotherapy.

Disclosure of Interests

The Authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Conflicts of Interest

None declared.

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Received October 13, 2014
Revised November 3, 2014
Accepted November 7, 2014