ANTICANCER RESEARCH *32*: 887-892 (2012)

Cytotoxicity of Activated Natural Killer Cells and Expression of Adhesion Molecules in Small-cell Lung Cancer

TADASHI TSUCHIDA[1], HIROMICHI YAMANE[2], NOBUAKI OCHI[2], TAKAYUKI TABAYASHI[2], AKIO HIRAKI[1], NAOYUKI NOGAMI[1], NAGIO TAKIGAWA[2], KATSUYUKI KIURA[3] and MITSUNE TANIMOTO[1]

[1]*Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Kita-ku, Okayama, Japan;*
[2]*Department of General Internal Medicine 4, Kawasaki Medical School, Kita-ku, Okayama, Japan;*
[3]*Department of Respiratory Medicine, Okayama University Hospital, Kita-ku, Okayama Japan*

Abstract. *Background/Aim: Although small-cell lung cancer (SCLC) is sensitive to anticancer agents, most patients with SCLC experience relapse and die within two years. Here, we examined the relationship between natural killer (NK) cells and adhesion molecules on SCLC cell lines. Materials and Methods: The expression levels of HLA class I, β2-microglobulin, Fas/Apo-1 receptor (FAS) and adhesion molecules on SCLC cell lines were examined by flow cytometry. The cytotoxicity of activated NK cells from SCLC patients was examined using ^{51}Cr-release assay. Results: HLA class I antigen and β2-microglobulin expression levels in SCLC cell lines were lower than those in healthy volunteers. SCLC cell lines were susceptible to lysis by activated NK cells but this showed no correlation with expression levels of adhesion molecules. Conclusion: Target cell susceptibility to activated NK cells from five SCLC patients correlated with survival benefit; target cell susceptibility to activated NK cells may be a surrogate marker of outcome for patients with SCLC.*

Lung cancer is the leading cause of cancer mortality in Japan and the majority of industrialized countries. While its incidence has decreased in the US, lung cancer is still increasing in many developing countries (1). Small-cell lung cancer (SCLC) is one of the solid tumors most sensitive to anticancer agents. Generally, platinum-based chemotherapy including etoposide or irinotecan is the mainstay of first-line treatment for SCLC. The objective response rate for SCLC with recent chemotherapy combinations exceeds 50%, even in patients with extensive disease, in Western countries (2). Despite high initial response rates, most patients eventually experience disease relapse. Except for topotecan and amrubicin, few treatment options remain after relapse (3, 4). Clinical trials of high-dose chemotherapy and autologous hematopoietic stem cell transplantation (AHSCT) showed that progression-free survival could be improved in patients with limited SCLC and in a few patients with extensive SCLC about a decade ago (5, 6); however, these positive results obtained in phase II clinical trials with high-dose chemotherapy were not confirmed in large-scale phase III trials. The SCLC chemotherapy status has not changed for 15 years, despite marked progress in molecular targeted therapy based on oncogenic driver mutation in non-SCLC (7). Accordingly, new approaches to improve SCLC treatment outcomes are needed. A meta-analysis of published randomized clinical trials involving maintenance therapy following systemic chemotherapy using cytokines showed no survival advantage (8). These results suggest that maintenance and/or the consolidation approach using interferons and other biological agents failed to improve SCLC outcomes. In an effort to investigate the possibility of an immunological approach using natural killer (NK) cells, we performed experiments to clarify the relationship between NK cell activity and adhesion molecules on SCLC cell lines.

Correspondence to: Hiromichi Yamane, MD, Ph.D., Department of General Internal Medicine 4, Kawasaki Medical School, 2-1-80 Nakasange, Kita-ku, Okayama 700-8505, Japan. Tel: +81 862252111, Fax: +81 862328343, e-mail: hiromichi.ya@gmail.com

Key Words: Natural killer cell, target cell susceptibility, HLA-class I, small-cell lung cancer, adhesion molecules.

Materials and Methods

Patients' characteristics. Table I shows the characteristics of our study population. All patients were diagnosed with SCLC pathologically and treated with standard- or high-dose chemotherapy with AHSCT. Disease stage was determined according to the UICC Manual of Clinical Oncology (9). All patients obtained a complete response. Performance status ranged from 0 to 4, with various clinical stages. Written informed consent concerning this study was obtained from all patients prior to treatment.

Table I. *Characteristics of patients with small-cell lung cancer participating in this study.*

Patient	Age (years)/Gender	PS	TNM	Stage	Treatment	Survival (years)	Relapse
1	53/M	4	T3N2M1	IV ED	HD-ICE	1.5	+
2	58/M	1	T4N3M0	IIIB LD	CT	3.4	+
3	75/M	0	T1N1M0	II LD	CT	0.8	+
4	50/M	1	T2N2M0	IIIA LD	CT	4.1	–
5	65/M	1	T4N2M0	IIIA LD	HD-ICE	4.5	–

ED, Extensive disease; LD, limited disease; PS, performance status; HDICE, high-dose chemotherapy (ifosfamide/carboplatin/etoposide) with autologous hematopoietic stem cell transplantation; CT, standard chemotherapy or chemoradiotherapy; TNM, tumor node metastasis.

Table II. *Characteristics of small-cell lung cancer cell lines.*

Cell line	Growth characteristic	Disease extent at diagnosis	Site of primary culture and disease status
SBC-1	Floating aggregates	T4N1M0, LD	Pleural effusion at PD
SBC-2	Loosely adherent	T2N2M1, ED	Bone marrow at PD
SBC-3	Loosely adherent	T3N2M1, ED	Bone marrow before treatment
SBC-4	Loosely adherent	T2N1M1, ED	Bone marrow at PD
SBC-5	Loosely adherent	T4N2M1, ED	Pleural effusion at PD
SBC-6	Loosely adherent	T1N2M1, ED	Pleural effusion at PD
SBC-7	Floating aggregates	T1N3M1, ED	Pericardial effusion at PD
SBC-9A	Floating aggregates	T2N2M1, ED	Pericardial effusion at PD
Lu-134-AH	Floating aggregates	Not reported	Primary lung tumor
NCI-H69	Floating aggregates	Not reported	Not reported
RERF-LC-FM	Floating aggregates	Not reported	Not reported

ED, Extensive disease; LD, limited disease; PD, progressive disease.

Cell lines. Table II summarizes the characteristics of 10 human SCLC lines examined in this study. SBC-1 (JCRB0816), SBC-2 (JCRB0817), SBC-3 (JCRB0818), SBC-4, SBC-5 (JCRB0819), SBC-6, SBC-7, SBC-9A (SCLC), EBC-2 (non-SCLC), and OU-LC-AS1 (non-SCLC) were established in our laboratories (10, 11). Lu-134-A-H (JCRB0235), RERF-LC-FM (JCRB0102), K562 (JCRB0019, a cell line from blast crisis of chronic myeloid leukemia), and a control NK-sensitive cell line were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). NCI-H69 was purchased from the American Type Culture Collection (Rockville, MD, USA). All cell lines were maintained in tissue culture flasks at 37°C under a humidified atmosphere supplemented with 5% CO_2 in air. The culture medium used in this study was RPMI-1640 (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals Japan Co., Ltd., Tokyo, Japan), penicillin-G (100 U/ml), and streptomycin (100 μg/ml).

Antibodies. Monoclonal antibodies (mAbs) used in this study were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-human HLA class I antigen mAb (clone Tu149, mouse IgG_{2a}) from Caltag Laboratories Inc. (Burlingame, CA, USA); phycoerythrin(PE)-conjugated anti-human CD56 mAb (clone B159, mouse IgG_1), anti-human β2-microgulobulin (clone Tu99, mouse IgM), PE-conjugated anti-human intercellular adhesion molecule 1 (ICAM-1) mAb (clone HA58, mouse IgG_1), and FITC-conjugated anti-Fas mAb from PharMingen (San Diego, CA, USA); and FITC-conjugated anti-human HLA class I molecule mAb (W6/32, clone B9.12.1, mouse IgG_{2a}) from Immunotech (San Francisco, CA, USA). Hybridoma secreting mAb NE150 (clone NB-1, mouse IgG_1) for CD56 (neural cell adhesion molecule, NCAM) was originally obtained from Dr. Takahashi (Aichi Cancer Center, Nagoya, Japan). NE150 was an ammonium sulfate precipitate of ascites, purified by protein A-Sepharose affinity chromatography. The NE150 $F(ab')_2$ fragments were prepared using Pierce ImmunoPure Fab Preparation Kit (Pierce Biotechnology Inc., Rockford, IL, USA).

Flow cytometry. Cells were incubated with each mAb and control mAb for 30 min at 4°C and were washed three times with phosphate-buffered saline (PBS) containing 0.2% FBS and 0.01% azide. For W6/32 and Tu99 mAbs, after washing, cells were incubated with FITC-conjugated F(ab') fragment goat anti-mouse IgG Ab for 20 min at 4°C and were washed three times with PBS containing 0.2% FBS and 0.01% azide. Flow cytometry was performed on a FACScan (Becton Dickinson, CA, USA), and the data were analyzed with CellQuest software (Becton Dickinson). To

quantify the expression levels of HLA class I molecules, NCAM, LFA-3, ICAM-1, FAS, and β2-microglobulin, the ratio of the mean fluorescence intensity (MFI) between test and control events was used (12). This ratio corresponded to the distance between the test and control MFI on a logarithmic scale. The expression levels of HLA class I molecules, β_2-microglobulin, adhesion molecules, and FAS were calculated as follows: 10^[log (MFI tested)]/10^[log (control MFI)] = 10^[log (MFI tested) – log (control MFI)].

Generation of effector cells. Table I shows the characteristics of patients with SCLC treated at Okayama University Hospital. Recombinant human interleukin-2 (rhIL-2, TGP-3; Takeda Chemical Industries, Ltd. Japan) activated NK cells were generated from peripheral blood mononuclear cells (PBMNCs) of untreated patients with SCLC, which were prepared by Ficoll-Hypaque and purified using Mini-MACS system (NK cell Isolation Kit, Miltenyi Biotec K.K., Japan). CD56-positive cells represented over 95% of cells following the purification procedure (data not shown). Purified NK cells ($1–1.5\times10^6$/ml) were cultured in RPMI-1640 supplemented with 10% FBS, non-essential amino acids, glutamine, sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, penicillin-G (100 U/ml), and streptomycin (100 μg/ml) (complete medium). A final concentration of 1000 U/ml rhIL-2 was added. On day 7, activated NK cells were used as effector cells.

Cytotoxicity assay. The prepared effector cells were first plated in 96-well, flat-bottomed, microplates in 150 μl/well and kept at 37°C under humidified atmosphere with 5% CO_2 in air prior to assay. Tumors cells as targets (1×10^6) were labeled with 100 μCi of sodium chromate (Na_2CrO_4) at 37°C for 45 min. The labeled target cells were washed twice with ice-cold RPMI-1640 containing 5% FBS. Target cells were added to the effector cells at 5000 cells/well in 50 μl immediately after the final wash. For cytotoxicity assay of tumor cells, several effector:target (E/T) ratios were used. The cytotoxicity test plates were incubated at 37°C under a humidified atmosphere with 5% CO_2 in air for 4 h. After incubation, the plates were centrifuged at 2000 rounds per minute for 5 min. For accuracy of the assay, the upper half of the supernatant fluid was used to count the release of ^{51}Cr using a gamma counter. The percentage of specific lysis was calculated as follows: % specific lysis=[(release tested cpm – spontaneous release cpm)/total release cpm] ×100.

Results

Expression level of HLA class I molecules on SCLC cell lines. Using flow cytometry, the expression of HLA class I molecules on SBC-7 cells was observed to be extremely low compared to those of lymphocytes from healthy volunteers or non-SCLC cells (Figure 1A). In addition, levels of HLA class I antigens and β_2-microglobulin in all SCLC cell lines were lower than those of lymphocytes from healthy volunteers (Figure 1B), which suggests that SCLC cells may be very susceptible to activated NK cells.

Cytotoxicity of activated NK cells against SCLC cell lines. Some previous reports had revealed that SCLC cells were sensitive to NK and lymphokine-activated killer (LAK) cells because HLA-class I antigen expression in SCLC cell lines was

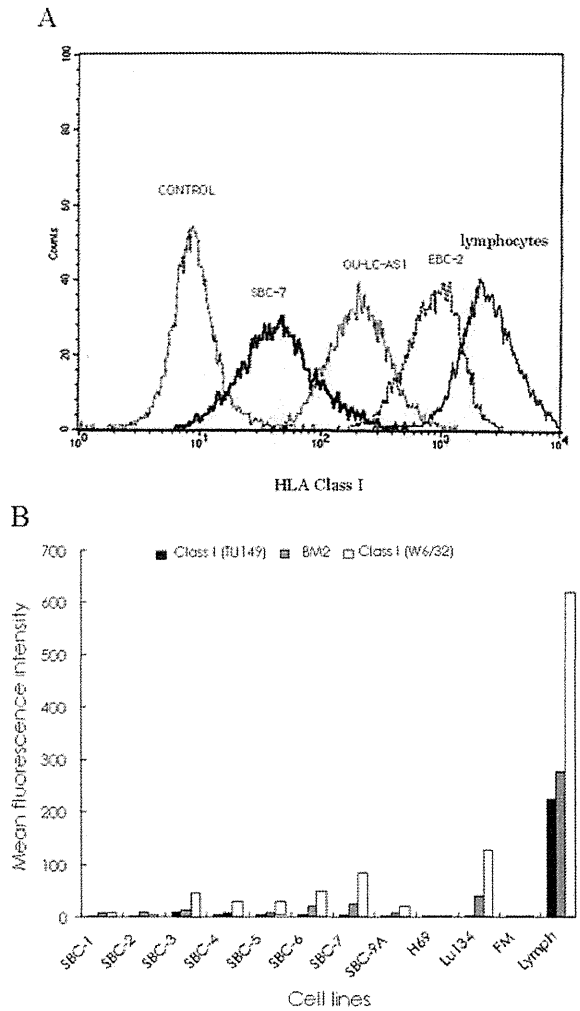

Figure 1. *A: Flow cytometric analysis of HLA class I molecules. The expression of HLA class I molecules on SBC-7 cells were extremely low compared with those of lymphocytes from healthy volunteers or non small-cell lung cancer cell lines (OU-LC-AS1 and EBC-2). B: Mean fluorescence intensity of HLA class I molecules and β_2-microglobulin by flow cytometric analysis. The expression levels of HLA class I molecules and β_2-microglobulin in all 11 small-cell lung cancer cell lines were extremely low compared with those of lymphocytes from healthy volunteers. H69: NCI-H69; Lu134: Lu-134-A-H; FM: RERF-LC-FM.*

equally supressed and NK and LAK cells do not receive negative signals through killer cell inhibitory receptor (KIR), such as p58 and p70, from down-regulating HLA class I molecules on SCLC cells (13, 14). Confirming this data, SCLC cells were found to be susceptible to activated NK cells generated from SCLC patients (Figure 2). Each SCLC cell line, except SBC-9a, was sensitive to activated NK cells and the NK-sensitive cell line K562. Moreover, activity against SCLC cell lines of NK cells from each patient differed in intensity.

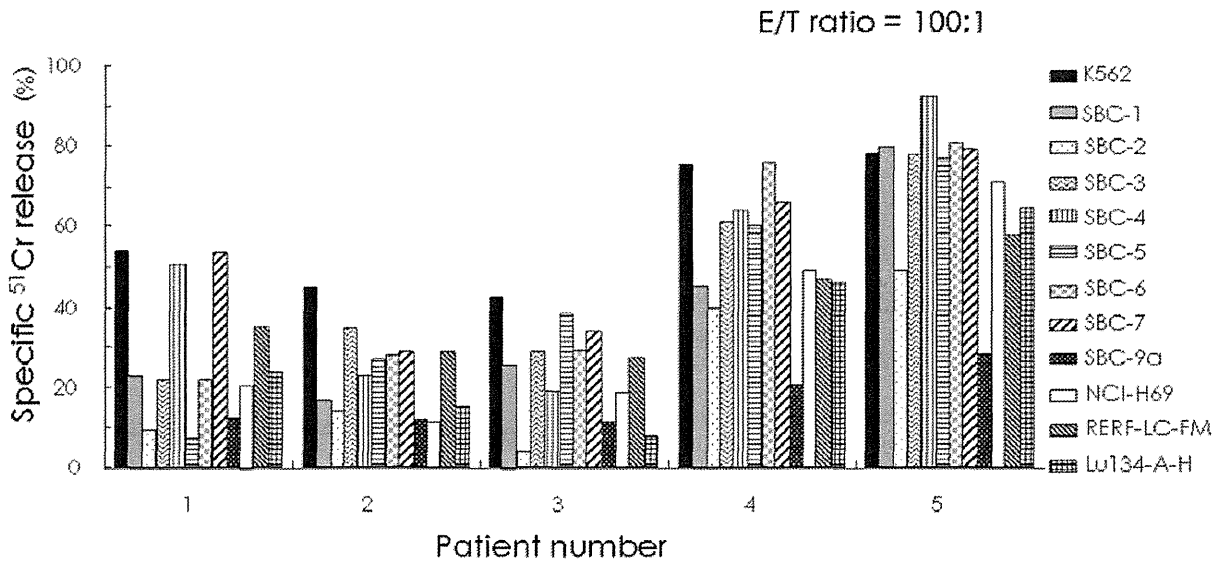

Figure 2. *Cytotoxicity of activated NK cells generated from SCLC patients against K562 and SCLC cell lines. Each SCLC cell line except SBC-9a as well as the cell line K562 were sensitive to lysis by activated NK cells.*

Relationship of adhesion molecules on SCLC cell lines and Cytotoxicity of NK cells towards SCLC cell lines. A previous report described CD56 on NK cells as a third pathway of cell adhesion other than those mediated by CD2/LFA-3 and LFA-1/ICAM-1 interactions and that CD56 was involved in NK cell cytotoxicity when interacting with cells bearing CD56/NCAM (15). In this experiment, the expression of NCAM, however, did not predict activated NK cell cytotoxicity (Figures 2 and 3A). In addition, the cytotoxicity of activated NK cells towards NCAM-overexpressing SBC-7 cells was not blocked even when anti-NCAM F(ab')2 mAb was added (Figure 4). Hence, we do not presume that NCAM–NCAM interaction mediated NK cell cytotoxicity towards SCLC cell lines. Differences in target cell susceptibility to activated NK cells showed no correlation with the expression level of HLA class I molecules, ICAM-1, or LFA-3 (16). We confirmed this finding that target cell susceptibility of activated NK cells showed no correlation with the expressions of ICAM-1, LFA-3, and FAS (Figure 3B).

Relationship of cytotoxicity towards SCLC cell lines and patient survival. Activated NK cells generated from patients 4 and 5 showed that the percentage specific ^{51}Cr-release was >40% in 10 out of 11 SCLC cell lines (Figure 2). These patients have survived more than 4 years without relapse. Activated NK cells generated from patients 2 and 3 showed that the percentage specific ^{51}Cr-release was ≤40% in all 11 SCLC cell lines, and ^{51}Cr-release of patient 1 was ≤40% in 9 out of 11 SCLC cell lines (Figure 2). These patients all experienced disease relapse and died.

Discussion

In this experiment, activated NK cells efficiently killed SCLC cells with a very low level of expression of HLA class I molecules. Furthermore, we confirmed previous data that target cell susceptibility to activated NK cells showed no correlation with the expressions of surface adhesion molecules. Target cell susceptibility to activated NK cells is very complex. Although the use of LAK cells has been intensively studied, this approach has not consistently shown benefits in clinical trials (17). Recent basic studies have shown that NK cells have a variety of receptors that recognize class I molecules. NK cell cytotoxicity is controlled by the balance between KIR and killer-cell activating receptor (KAR). NK cells can kill the allografts that are lacking one or more self-class I molecules because no negative signals are transmitted to a particular subset of NK cells through KIR (18). Several reports have shown that HLA-class I molecule expression levels in lung cancer cell lines are reduced and these molecules are susceptible to lysis by NK and LAK cells (12, 13).

In high-dose chemotherapy treatments with AHSCT, peripheral blood stem cells (PBSCs) contain more NK cells than do bone marrow aspirates (19). If these NK cells are effectively activated, contaminating tumor cells in PBSCs should be eradicated. Thus, we assume that a very small number of SCLC cells are susceptible to lysis by NK cells or activated NK cells. Even recent and very intensive therapy will be unable to eradicate all tumor cells. Presumably, between 10^5 and 10^6

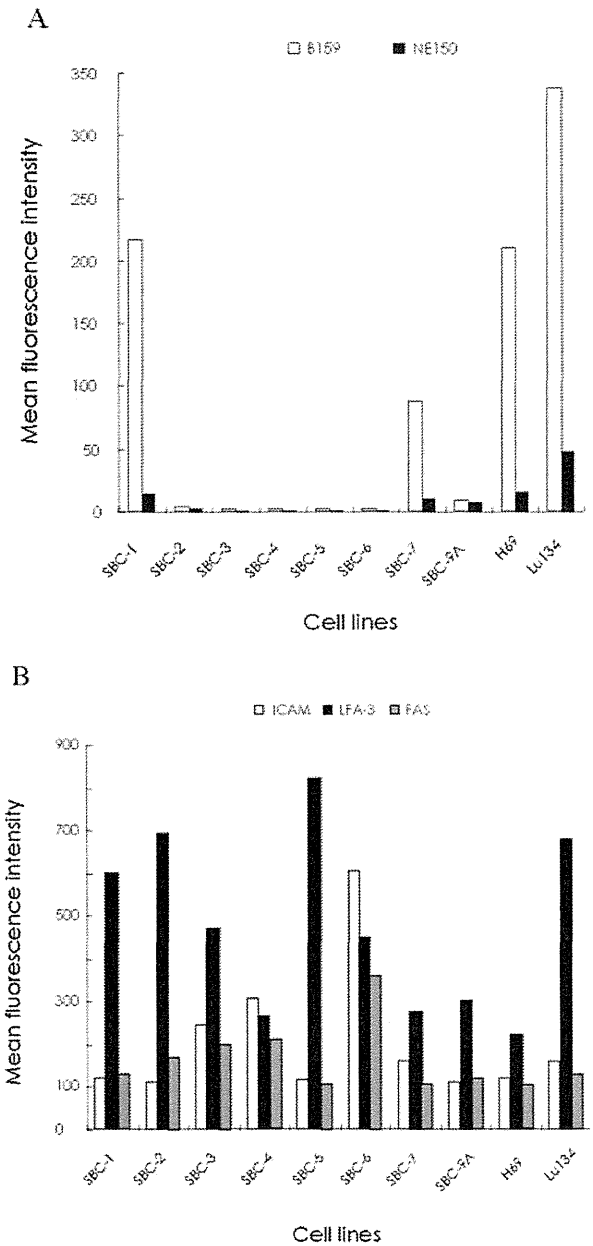

Figure 3. *Expression of neural cell adhesion molecule (NCAM) (A) and Expression of intercellular adhesion molecule (ICAM), lymphocyte function-associated antigen-3 (LFA-3) and Fas/Apo-1 receptor (FAS) (B) on small-cell lung cancer cells by flow cytometric analysis.*

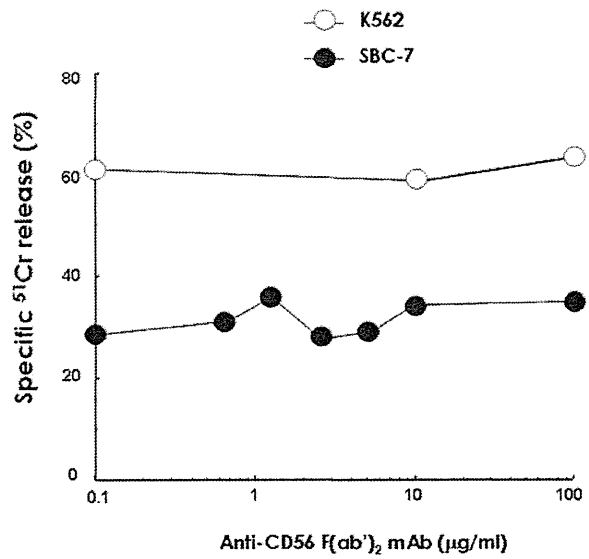

Figure 4. *Effects of anti-CD56 F(ab')$_2$ mAb on cytotoxicity of activated natural killer (NK) cells against K562 and SBC-7 cells. The cytotoxicity of activated NK cells on neural cell adhesion molecule (NCAM)-overexpressing SBC-7 cells was not blocked even when anti-NCAM F(ab')$_2$ mAb was added.*

tumor cells remain even in complete responders. If complete responders achieve a cure, minimal residual SCLC cells are eradicated efficiently by NK cells or activated NK cells.

Strong cytotoxicity of activated NK cells against SCLC cell lines may be linked to patients' immune status after intensive treatment. In our results, patients with specific ^{51}Cr-release >40% in 10 out of 11 SCLC cell lines have survived without disease and are working full-time. However, patients with specific ^{51}Cr-release ≤40% in the majority of the SCLC cell lines experienced disease relapse and died. This result indicates that target cell susceptibility to activated NK cells from patients with SCLC may correlate with favorable clinical outcomes. In other words, the target cell susceptibility to activated NK cells may be a surrogate marker of clinical outcome of SCLC patients. Clinical trials of high-dose chemotherapy with AHSCT and the use of LAK cells have not consistently shown an apparent benefit in unselected SCLC patients. However, these modalities could be one of the standard treatments if the benefits were proven in clinical trials in selected SCLC patients using target cell susceptibility to activated NK cells.

In conclusion, since the cytotoxicity of activated NK cells towards SCLC cell lines may be linked to the immune state after intensive treatment, SCLC patients achieving a complete response should be exposed to a promoter of NK activity. Prospective trials will be essential to elucidate the role of NK cells or activated NK cells prior to adopting new treatments.

Acknowledgements

We thank Masahiro Tabata, Hiroshi Ueoka and Mine Harada, Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, for helpful comments and discussion during drafting of this report.

References

1 Jemal A, Siegel R, Ward E, Hao Y, Xu J and Thun MJ: Cancer statistics. CA Cancer J Clin *59*: 225-249, 2009.

2 Hanna N, Bunn PA Jr., Langer C, Einhorn L, Guthrie T Jr, Beck T, Ansari R, Ellis P, Byrne M, Morrison M, Hariharan S, Wang B and Sandler A: Randomized phase III trial comparing irinotecan/cisplatin with etoposide/cisplatin in patients with previously untreated extensive stage disease small-cell lung cancer. J Clin Oncol *24*: 2038-2043, 2006.

3 van Meerbeeck JP, Fennell DA and De Ruysscher DK: Small-cell lung cancer. Lancet *378(9804)*: 1741-1755, 2011.

4 Inoue A, Sugawara S, Yamazaki K, Maemondo M, Suzuki T, Gomi K, Takanashi S, Inoue C, Inage M, Yokouchi H, Watanabe H, Tsukamoto T, Saijo Y, Ishimoto O, Hommura F and Nukiwa T: Randomized phase II trial comparing amrubicin with topotecan in patients with previously treated small-cell lung cancer: North Japan Lung Cancer Study Group Trial 0402. J Clin Oncol *26(33)*: 5401-5406, 2008.

5 Elias A, Ibrahim J, Skarin AT, Wheeler C, McCauley M, Ayash L, Richardson P, Schnipper L, Antman KH and Frei E 3rd: Dose-intensive therapy for limited-stage small-cell lung cancer: long-term outcome. J Clin Oncol *17*: 1175-1184, 1999.

6 Elias AD, Skarin AT, Richardson P, Ibrahim J, McCauley M and Frei E 3rd: Dose-intensive therapy for extensive-stage small cell lung cancer and extrapulmonary small cell carcinoma: long-term outcome. Biol Blood Marrow Transplant *8*: 326-333, 2002.

7 Costanzo R, Piccirillo MC, Sandomenico C, Carillio G, Montanino A, Daniele G, Giordano P, Bryce J, De Feo G, Di Maio M, Rocco G, Normanno N, Perrone F and Morabito A: Gefitinib in non small cell lung cancer. J Biomed Biotechnol *2011*: 815269, 2011.

8 Rossi A, Garassino MC, Cinquini M, Sburlati P, Di Maio M, Farina G, Gridelli C and Torri V: Maintenance or consolidation therapy in small-cell lung cancer: a systematic review and meta-analysis. Lung Cancer *70(2)*: 119-128, 2010.

9 Payne D, Burkes R and Ginsberg RJ: Chapter 17. Lung Cancer. *In*: UICC manual of clinical oncology. 8th ed. Pollock RE, Doroshow JH, Khayat D, Nakao A and O'Sullivan B (eds.). .Hoboken, New Jersey, Wiley Liss, pp. 381-403, 2004.

10 Kawai H, Kiura K, Tabata M, Yoshino T, Takata I, Hiraki A, Chikamori K, Ueoka H, Tanimoto M and Harada M: Characterization of non-small cell lung cancer cell lines established before and after chemotherapy. Lung Cancer *35*: 305-314, 2002.

11 Hiraki A, Kaneshige T, Kiura K, Ueoka H, Yamane H, Tanaka M and Harada M: Loss of HLA haplotype in lung cancer cell lines: implications for immunosurveillance of altered HLA class I/II phenotypes in lung cancer. Clin Cancer Res *5*: 933-936, 1999.

12 Hiraki A, Fujii N, Murakami T, Kiura K, Aoe K, Yamane H, Masuda K, Maeda T, Sugi K, Darzynkiewicz Z, Tanimoto M and Harada M: High frequency of allele-specific down-regulation of HLA class I expression in lung cancer cell lines. Anticancer Res *24*: 1525-1528, 2004.

13 Tanio Y, Watanabe M, Osaki T, Tachibana I, Kawase I, Kuritani T, Saito S, Masuno T, Kodama N, Furuse K and Kishimoto T: High sensitivity to peripheral blood lymphocytes and low HLA-class I antigen expression of small cell lung cancer cell lines with diverse chemo-radiosensitivity. Jpn J Cancer Res *83*: 736-745, 1992.

14 Lagadec PF, Saraya KA and Balkwill FR: Human small-cell lung cancer cells are cytokine-resistant but NK/LAK-sensitive. Int J Cancer *48*: 311-317, 1991.

15 Nitta T, Yagita H, Sato K and Okumura K: Involvement of CD56 (NKH-1/Leu-19 antigen) as an adhesion molecule in natural killer target cell interaction. J Exp Med *170*: 1757-1761, 1989.

16 Lehmann C, Glass B, Zeis M, Schmitz N and Uharek L: Investigating the lysis of small-cell lung cancer cell lines by activated natural killer (NK) cells with a fluorometric assay for NK cell-mediated cytotoxicity. Cancer Immunol Immunother *48*: 209-213, 1999.

17 Yamaguchi Y, Ohshita A, Kawabuchi Y, Ohta K, Shimizu K, Minami K, Hihara J, Miyahara E and Toge T: Adoptive immunotherapy of cancer using activated autologous lymphocytes-current status and new strategies. Hum Cell *16*: 183-189, 2003.

18 Purdy AK and Campbell KS: Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR). Cancer Biol Ther *8*: 2211-2220, 2009.

19 Majolino I, Cavallaro AM and Scimè R: Peripheral blood stem cells for allogeneic transplantation. Bone Marrow Transplant *18(2)*: 171-174, 1996.

Received January 20, 2012
Revised February 16, 2012
Accepted February 17, 2012